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Preface

The present book is a compilation of high-quality contributions centered around elucidating the underlying molecular pathways to uncover new avenues for therapy and diagnosis in the fields of immunology, inflammation, cancer, endocrinology, and several others.

Its conceptualization emerges as a joint effort from researchers belonging to The Millennium Institute on Immunology and Immunotherapy (MIII) to encourage outreach within the research community at a global scale, with articles submitted by renowned investigators worldwide.

The MIII is a center for scientific excellence that brings together researchers from six Chilean universities: the Pontificia Universidad Católica de Chile, Universidad de Chile, Universidad Andrés Bello, Universidad de la Frontera, Universidad de Antofagasta, and Universidad Austral. At MIII, basic immunology research is performed to obtain practical applications in immunotherapy, such as new immunological therapies, vaccines, and new pharmacological tools. Research conducted at MIII is aimed at training young scientists and fully understanding the operation of the immune system to develop new therapies to cope with human diseases related to cancer, autoimmunity, infections, cardiovascular pathologies, and endocrine disorders. Since 2015, the MIII has been a formal member of the Center Excellence Network belonging to the Federation of Clinical Immunology Societies, which includes several centers in the USA, Europe, and Asia. The MIII was the first FCE in the southern end of America.

We thank each of the individual researchers and their groups for contributing with hot-topic articles bringing balance and diversity to this work. Special acknowledgments to Dr. Sebastian Gatica for his extraordinary support and assistance in preparing every detail of this book.

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Part I

Molecular Pathology of Immune, Inflammatory, and Hemostatic Disorders
Immune Responses at Host Barriers and Their Importance in Systemic Autoimmune Diseases

Katina Schinnerling, Hugo A. Penny, Jorge A. Soto, and Felipe Melo-Gonzalez

Abstract

Host barriers such as the skin, the lung mucosa, the intestinal mucosa and the oral cavity are crucial at preventing contact with potential threats and are populated by a diverse population of innate and adaptive immune cells. Alterations in antigen recognition driven by genetic and environmental factors can lead to autoimmune systemic diseases such as rheumatoid arthritis, systemic lupus erythematosus and food allergy. Here we review how different immune cells residing at epithelial barriers, host-derived signals and environmental signals are involved in the initiation and progression of autoimmune responses in these diseases. We discuss how regulation of innate responses at these barriers and the influence of environmental factors such as the microbiota can affect the susceptibility to develop local and systemic autoimmune responses particularly in the cases of food allergy, systemic lupus erythematosus and rheumatoid arthritis. Induction of pathogenic autoreactive immune responses at host barriers in these diseases can contribute to the initiation and progression of their pathogenesis.

Keywords

Autoimmunity · Host barriers · Food allergy · Systemic lupus erythematosus · Rheumatoid arthritis

Abbreviations

ACPA Anti-citrullinated protein antibodies
HLA Human leukocyte antigen
HSD High-salt diet
IBS Irritable bowel syndrome
IFN-γ Interferon gamma
Ig Immunoglobulin
IL Interleukin
ILC Innate lymphoid cell
ILD Interstitial lung disease
LPS Lipopolysaccharide
LBP LPS-binding protein

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1.1 Introduction

Epithelial surfaces constitute an important physical barrier, which protects the body from external threats, but also provides an interface for communication between host and environment. The intercellular space between epithelial cells is sealed by tight junctions. These are complex structures composed of several proteins, including occludin, claudins, and zonula occludens, which regulate the permeability of the epithelium to small molecules [1]. In addition, mucosal barriers such as the lung and the intestine are covered by complex glycoproteins called mucins, protecting the epithelial layer from microbial colonization and exposure to environmental signals [2]. However, the intestine plays a key role at absorbing different nutrients and thus a fine regulation between tolerance and defensive responses needs to be achieved. Innate immune cells are key regulators of the initiation of pro-inflammatory responses upon stimulation with environmental antigens at epithelial barriers. Environmental stress, infections, microbial dysbiosis, pro-inflammatory cytokines, and diet can alter the permeability of the epithelial barrier, which might have systemic consequences, including the development of hypersensitivity and autoimmune disorders in susceptible individuals. This review focuses on the pivotal role of immune responses at epithelial barriers for the initiation and progression of systemic inflammatory diseases, such as food allergy, systemic lupus erythematosus, and rheumatoid arthritis.

1.2 Food Allergy

Food allergies are usually characterized by exacerbated type 2 immune responses that can lead to systemic inflammation and potentially fatal anaphylactic reactions. Recent studies suggest that 10.8% of US adults have a food allergy but this percentage may be even higher, as 19% of US adults self-reported a food allergy in a recent population-based survey [3]. Importantly, a high percentage of individuals reporting food allergies exhibit severe reactions and/or exhibit multiple food allergies [3]. In addition, food allergy is more prevalent in women and some ethnic groups based in the electronic health records of a large population group in US but peanut allergy may be more prevalent in men [4]. Although prevalent in westernized countries, several aspects of the pathogenesis of food allergies are incompletely understood, due to the inability to replicate them in animal models. This section will explore some of the described mechanisms of initiation of food allergy at the intestinal mucosa and the skin, which have been mainly characterized in murine models of allergy using the chicken egg allergen ovalbumin (OVA) and other dietary antigens.

1.2.1 Initiation of Food Allergy at the Intestinal Mucosa

Sensitization to food allergens has been proposed to start at the intestinal mucosa, by the uptake of dietary allergens and subsequent antigen presentation followed by the induction of allergen-specific T helper type 2 (Th2) cells and allergen-specific Immunoglobulin (Ig)E secretion (reviewed in [5]). Further exposure to the allergen may induce binding of IgE to FcεR receptors on the surface of mast cells and basophils, leading to degranulation and release of inflammatory...
mediators such as histamine [5]. In this way, ingestion of food allergens may lead to serious adverse systemic reactions and anaphylaxis.

The type 2 cytokines interleukin (IL)-4 and IL-13 have been studied as potential initiators of food allergy in the intestinal mucosa. Mice exhibiting enhanced IL-4 receptor signaling develop anaphylaxis following OVA sensitization in the presence or absence of bacterial toxins. These mice exhibited increased intestinal permeability, intestinal mast cell hyperplasia, Th2 responses and OVA-specific IgE. Lack of IgE or the IgE receptor FcεRII prevented anaphylaxis in this mouse model, indicating that IL-4 signaling may induce IgE-dependent anaphylaxis to food allergens [6]. In contrast, mice deficient in IL-4 production sensitized to OVA exhibit low levels of IgE, reduced intestinal mast cell degranulation and resistance to OVA-induced diarrhea, whereas IL-13 deficient mice were partially protected against diarrhea. Importantly, prophylactic administration of an anti-IL-4Ra antibody in WT mice sensitized to OVA was protective against OVA-induced diarrhea, its administration after the sensitization reduced IgE and mast cell degranulation, but failed to reduce allergen-induced diarrhea, suggesting that IL-4 is not required at the effector phase of the response [7].

IL-4 has been proposed as a key regulator of intestinal mast cells during food allergy, as it is able to induce intestinal mastocytosis. Enhanced IL-4 receptor signaling drives enhanced anaphylactic reactions in a model of passive sensitization with anti-2,4-dinitrophenol (DNP) IgE and challenge with DNP-BSA (bovine serum albumin). In addition, IL-4-Ra signaling triggers intestinal mast cell expansion during OVA-induced allergy, which may be mediated by STAT6 signaling and subsequent increased levels of the high affinity receptor FcεR II, improved mast cell survival and activation [8]. Several studies have shown that sensitization to OVA increases intestinal mast cells and a higher density of these cells correlates with the severity of systemic symptoms in IgE-mediated oral antigen-induced anaphylaxis [9]. In addition, transgenic mice overexpressing intestinal-specific IL-9 are more susceptible to develop food allergy and IgE-mediated anaphylaxis, as they exhibit intestinal mastocytosis and increased intestinal permeability [10]. Importantly, a population of IL-9-producing mucosal mast cells are the main source of IL-9 in a model of IgE-mediated food allergy and they arise in response to IL-4-producing Th2 cells, amplifying intestinal mastocytosis [11].

On the other hand, other innate immune cells have been implicated in the pathogenesis of food allergy. In a model of intraperitoneal OVA-sensitization and intragastric OVA challenge, in vivo basophil depletion prevented allergic diarrhea but did not alter systemic total IgE and anti-OVA IgE levels, indicating that basophils may play a role during the effector phase, but not during the priming phase, of food allergy [12]. Selective and inducible deletion of mast cells and basophils in mice results in decreased peanut-induced anaphylaxis, indicating that both cell types are involved in their pathogenesis [13]. Group 2 innate lymphoid cells (ILC2s) are also important sources of the type 2 cytokines IL-4, IL-5 and IL-13 and are induced upon stimulation with different alarmins such as IL-33 and IL-25 [14]. Intestinal ILC2 may activate in response to mast cell-derived signals during experimental food allergy using OVA or peanut allergens [15]. Mice lacking mast cells or IgE exhibit impaired ILC2 activation during food allergy and IgE-activated mast cell drive ILC2 expansion in an IL-4Ra-dependent manner [15]. In addition, ILC2-derived IL-13 can increase susceptibility to severe anaphylaxis by enhancing responsiveness to mast cell-derived signals [15]. Expression of IL-25 during experimental food allergy precedes anaphylaxis and induces IL-5 and IL-13 expression on intestinal ILC2s, as shown in transgenic mice overexpressing IL-25 or lacking the IL-25 receptor IL-17RB [16]. ILC2-derived IL-13 is also enhanced by allergen-specific CD4+ Th2 cells expressing IL-17RB in response to IL-25 and mice lacking CD4+ Th2 cells do not exhibit ILC2-mediated food allergy [16]. Therefore, complex interactions between several immune
cell types may be important for both priming and effector phases of food allergies (Table 1.1).

Enteric infections may participate in the local induction of allergic responses. Mice infected with *Citrobacter rodentium*, or exposed to the superantigen staphylococcal enterotoxin B, exhibit an increase in intestinal IgE antibodies against dietary antigens in murine models [17]. Further exposure to these dietary antigens leads to visceral pain in an IgE- and mast-cell dependent mechanism, which subsequently leads to increased histamine release and sensitization of visceral afferents [17]. It was further demonstrated that patients with irritable bowel syndrome (IBS) exhibited antigen-specific IgE responses, mast cell activation and inflammation following local administration of dietary antigens (soy, wheat, gluten, milk) into the GI tract [17]. Although these responses were restricted to the gastrointestinal tract, clinical evidence suggests an overlap between gastrointestinal disorders such as IBS and atopic diseases including asthma, eczema, and dermatitis [18, 19].

### 1.2.2 Initiation of Food Allergy at the Skin Barrier

On the other hand, several studies have linked the pathogenesis of food allergies with skin damage and skin allergies. Patients with atopic dermatitis exhibit high prevalence of IgE antibodies against food allergens and display increased numbers of intestinal IgE+ cells and increased intestinal permeability [20, 21]. It has been hypothesized that defects in the epithelial barrier may contribute to food allergen sensitization. Loss-of-function mutations in the gene encoding the skin polyprotein filaggrin (FLG) show a strong correlation with IgE-mediated peanut allergy in food-challenge positive patients [22]. In addition, exposure to environmental peanut dust has been identified as a risk factor for peanut allergy in children carrying FLG mutations, indicating that peanut sensitization in the skin can occur during the infancy [23]. Therefore, alterations in the skin barrier in susceptible individuals may contribute to the initiation of food allergy.

Murine models of sensitization have revealed some of the mechanisms that may induce food allergy following skin exposure to the allergen. Repeated cutaneous exposure to OVA in a mouse model led to increased levels of IL-4 in circulation, expansion of intestinal mast cells and IgE-dependent anaphylaxis. In contrast, mice treated orally with OVA in combination with cholera toxin for 8 weeks exhibited an increased in serum IgE but not the other parameters and subsequently did not develop anaphylaxis following an oral challenge with OVA [24]. In addition, epicutaneous exposure and challenge with OVA induces increased IL-4 secretion and induction of IL-9-producing mucosal mast cells, which is mediated by IL-4Ra receptor and partly dependent on BATF signaling on mast cells [25]. These results indicate that skin exposure to food antigens may lead to abnormal intestinal immune responses and later to systemic allergic responses.

An important epithelial-derived signal able to induce type 2 responses is thymic stromal lymphopoietin (TSLP). In a model of skin lesion followed by epicutaneous sensitization with OVA or peanut allergen, TSLP drives an increase of skin basophils and subsequent increase in antigen-specific secretion of IL-4 and IL-5 in the skin lymph nodes, antigen-specific serum IgE and accumulation of mast cells in the intestine [26]. Food allergy susceptibility was reduced by disruption of TSLP responses or basophil depletion, indicating that the TSLP-basophil axis may be important in the induction of food allergy in damaged skin and antigen sensitization [26]. In a similar model, TSLP requires TSLPR-signaling on dendritic cell (DC) to induce Th2 responses in the intestine [27]. Experiments using TSLP-deficient mice or TSLP blocking showed that local TSLP is sufficient to drive food allergy but systemic TSLP is not required to induce food allergy and may be required in the sensitization phase but not in the challenge [27]. Therefore, TSLP can promote the initiation of allergic responses through basophil and DC activation and drive food allergy.
| Autoimmune disease | Host barrier | Environmental triggers | Epithelial-derived factors | Microbial-derived factors | Immune factors | Systemic effects |
|---------------------|--------------|------------------------|---------------------------|--------------------------|----------------|-----------------|
| **Food allergy**    | Skin         | Skin damage + allergen sensitization | TSLP, IL-33 | – | † ILC2 (IL-4, IL-5, IL-13) | IL-4, IgE-mediated anaphylaxis |
|                     |              |                        |                           |                          | † Tuft cells (IL-25) |               |
|                     |              |                        |                           |                          | † Basophils (IL-4) |               |
|                     |              |                        |                           |                          | † Mast cells (IL-9) |               |
| **Intestine**       |              | Allergen sensitization | IL-33, IL-25 | – | † ILC2 (IL-4, IL-5, IL-13) | IgE-mediated anaphylaxis |
|                     |              |                        |                           |                          | † Basophils (IL-4) |               |
|                     |              |                        |                           |                          | † Mast cells (IL-9) |               |
|                     |              | Dysbiosis (↓ Clostridiales) + allergen sensitization | Unknown | Metabolites? | Th2-like T<sub>reg</sub> (IL-4) |               |
| **Systemic lupus erythematosus** | Skin | † *Staphylococcus aureus* | Unknown | Superantigens/toxins? | Neutrophils (NETs) | SLE-associated autoantibodies (anti-dsDNA, anti-Sm) |
|                     |              |                        |                           |                          | Th17 cells | Ig immunocomplex |
| **Intestine**       |              | Dysbiosis (↓ Firmicutes ↑ Bacteroides) | Unknown | LPS | Th17 cells | LPS, CD14, and α1-acid glycoprotein |
|                     |              | Intestinal permeability | ↑ Tight junctions | LPS | Th17 cells? | LPS, CD14, and α1-acid glycoprotein |
| **Rheumatoid arthritis** | Oral cavity | Keystone pathogens (*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans*) | Unknown | PPAD LtxA | Neutrophil (NETs), Th17 cells (IL-17) | ACPA |
|                     |              | Dysbiosis (↑ Prevotella sp., *Anaeroglobus geminatus*) | IL-33 | LPS/others? | Neutrophil (NETs), Th17 cells (IL-17), Th1 cells (IFN-γ, TNF-α) | ACPA, RF |
|                     | Lung         | Tobacco smoke, inhaled pollutants, dysbiosis | Unknown | – | Neutrophil (NETs) | ACPA |
|                     | Intestine    | Dysbiosis (↑ *Prevotella copri*, ↑ *Collinsella aerofaciens*, ↑ *Subdoligranulum*) | Unknown | LPS/others? | Th17 cells (IL-17) | Autoreactive T cells (Th1, Th17), IgG and IgA autoantibodies |
|                     |              | Dysbiosis (↑ SFB) | Unknown | ↓ SCFA | Thf cells, ↓ IL-2 | Autoantibodies |
|                     |              | Intestinal permeability | ↓ Tight junctions | LPS | ↓ IL-10R | LPS, LBP, sCD14 |

*Abbreviations*: ACPA anti-citrullinated protein antibodies; IL interleukin; ILC innate lymphoid cells; LPS lipopolysaccharide; LBP LPS-binding protein; Ltx-A leukotoxin-A; NET neutrophil extracellular traps; PAD peptidyl-arginine deiminase; RF rheumatoid factor; SCFA short chain fatty acids; SFB segmented filamentous bacteria; SLE systemic lupus erythematosus; Th T helper cells
Extensive evidence suggest that IL-33 is also an important inducer of food allergy following antigen sensitization in damaged skin. In line with this, a murine model of sustained mechanical skin injury resulted in increased local and systemic production of IL-33 and mice deficient in the IL-33 receptor ST2 exhibited less severity in a model of oral anaphylaxis to OVA. These effects were attributed to the stimulatory role of IL-33 in IgE-mediated mast cell degranulation, as mice deficient in mast cells were protected against oral anaphylaxis [28]. Another similar study showed that OVA epicutaneous sensitization in a model of skin lesion induced keratinocyte-derived IL-33, inducing food allergy in a TSLP-independent manner but it is unclear whether TSLP could directly induce IL-33 production in the skin [29]. These findings show an important role of keratinocyte-derived IL-33 during the initiation and progression of food allergy. Furthermore, a role for skin-derived IL-33 and gut-derived IL-25 in the activation of ILC2 has been reported in the same model of skin injury [30]. Skin injury induced a systemic release of IL-33 which resulted in expansion in the expansion and activation of intestinal ILC2, which secrete IL-4 and IL-13, leading to an expansion of intestinal mast cells and an increase in intestinal permeability [30]. In addition, ILC2-derived IL-13 drives the expansion of Tuft cells and subsequent release of IL-25, which synergizes with IL-33 in intestinal ILC2 activation. Thus, mice with skin injury and subjected to an oral model of anaphylaxis with OVA exhibited more severe allergic reactions [30]. These findings suggest that the alterations in intestinal mast cells driven by skin injury may lead to altered intestinal permeability and could subsequently induce higher antigen absorption. IL-33 has been also associated with sensitization to the peanut allergens Ara h 1 and Ara h following epicutaneous exposure to peanut in mice [31]. Keratinocyte-derived IL-33 may modulate DC function and, in turn, drive Th2 priming, which was significantly reduced following anti-ST2 blockage in vivo [31]. Therefore, IL-33 acts as an important signal promoting allergic responses systemically (Table 1.1).

A model of murine long-term skin depilation (hairlessness) also showed alterations in skin permeability together with induction of the inflammasome pathway, characterized by increased expression of the receptor NLRP3 and secretion of the pro-inflammatory cytokines IL-1b and IL-18 [32]. Following long-term skin depilation, mice were epicutaneously sensitized and challenged with the major peach allergen Pru p 3 and developed skin inflammation, infiltration of ILC-like lymphocytes into the skin and anaphylaxis [32]. In line with this, patients with peach allergy exhibited increased ILC2 in circulation whereas incubation with Pru p 3 induced higher levels of the co-stimulatory molecule CD86 and sphingosine-1-phosphate receptor 1, suggesting that ILC2 may play a role in the induction of peach allergy [32]. However, it remains to be elucidated in peanut and peach allergy whether ILC2s migrate from the skin to the gut after allergen sensitization and which mechanisms may regulate their migration.

1.2.3 Mechanisms of Oral Tolerance in Food Allergy

Several mechanisms related to immunological tolerance prevent food allergy. A recent study demonstrated that ingestion of a peanut butter product in mice may be protective in a model of airway or skin sensitization to peanut. Peanut-specific IgE and IgG antibodies were abrogated following peanut ingestion, which was mediated by a population of cytotoxic T lymphocyte-associated protein 4 (CTLA-4)+ CD4+ T regulatory cells, preventing germinal center B cell allergic responses [33]. Similarly, mice orally exposed to OVA before skin sensitization exhibited reduced food allergy and were protected from anaphylaxis [27]. These findings suggest that oral tolerance may be able to counteract allergic responses initiated by allergen sensitization in the skin.

Antigen-specific IgA may play an important inhibitory role in allergy, as shown in a study using mouse bone marrow derived-mast cells and peritoneal mast cells, preventing IgE-mediated
degranulation in an allergen-specific manner [34]. This inhibition of mast cell-allergic functions is mediated by the suppression of Syk phosphorylation. In addition, IgA prevented peanut-induced activation in basophils from a peanut allergic donor, which suggests that IgA may control basophil and mast cell function at mucosal barriers [34]. Indeed, a potential role of IgA preventing food allergen uptake at the intestinal mucosa by M cells has been proposed as a mechanism of oral tolerance [35]. In addition, a model of IgE-mediated anaphylaxis was prevented by circulating IgA antibodies rather than locally secreted mucosal IgA as it was shown that in this model the allergen needs to be absorbed systemically to induce IgE-mediated anaphylaxis [36].

T regulatory (Treg) cells play an important role preventing autoimmune responses in food allergy. Treg cell-derived transforming growth factor (TGF)-β1 can prevent mast cell expansion and activation, which may prevent food allergy [37]. In contrast, allergen-specific pathogenic Treg cells acquire a Th2-like phenotype secreting IL-4 during experimental food allergy, indicating that alterations in Tregs may be involved in food allergy [38]. On the other hand, Treg cell-derived TGF-β1 can also induce the differentiation of RAR-related orphan nuclear (ROR)γt+ Treg cells, which induce oral tolerance in response to Clostridiales and Bacteroidales during mouse weaning and human infancy [37]. Indeed, treatment with bacterial consortium of both groups prevented experimental food allergy by inducing RORγt+ Treg cells in a Myd88-dependent manner [37, 39]. In addition, Clostridia species may induce IL-22 expression on ILC3 and T cells, which reinforces the intestinal barrier and reduces allergen access to the bloodstream [40]. In line with this, gut dysbiosis may be associated with increased susceptibility to food allergy and anaphylaxis in mice, by either the expansion of pathogenic microorganisms such as Enterococcus and/or the reduction of beneficial species including Clostridiales and Akkermansia that contribute to altered intestinal permeability and allergic immune responses in murine models (Table 1.1) [41–44]. Indeed, consumption of antibiotics or high fat diet causes gut dysbiosis and increases susceptibility to experimental food allergy [43, 44]. Therefore, microbiota-induced Tregs and other protective signals induced by the microbiota may be crucial at preventing food allergy, but whether augmentation of the commensal niche with probiotic supplements promotes immunological tolerance towards dietary allergens and/or improves symptoms associated with food allergy remains unclear [45, 46].

1.3 Systemic Lupus Erythematosus and Its Association with Mucosal Barriers

Systemic lupus erythematosus (SLE) is an inflammatory and multi-systemic disease characterized by the immune system’s attack on its own tissues. Mainly, this disease is triggered by the generation of autoantibodies, immune complexes, and an aberrant response of the adaptive immune system cells [47]. Among the main affected organs, we find the heart, kidneys, lungs, joints, bones, and skin [48]. Additionally, SLE has been related to the appearance of mucosal damage directly associated with oral and gastrointestinal lesions [49–51].

About 40% of patients with SLE may manifest oral lesions, with oral ulcers being the most identified in them [52]. However, these manifestations can be asymptomatic in many cases, dismissing this variable for the patient’s clinical diagnosis, which is one of the four criteria for the diagnosis of SLE [53]. Oral lupus erythematosus (OLE) is a complex condition presented as interface mucositis associated with hyperkeratosis and an alternation between epithelial hyperplasia, atrophy, and other multiple biological processes [53]. At the immunological level, an increase in deposits of IgG, IgA, and IgM immunoglobulins, together with the C3 protein of the complement pathway, are identified in the areas of the lesion [52, 53].
1.3.1 Dysbiosis of Intestinal Microbiota and “Leaky Gut” as Triggers of Inflammation in SLE

Currently, more evidence suggests that different degrees of dysbiosis in mouse models and patients with SLE may be related to intestinal infection, thus promoting a more significant development of SLE. SLE-patients exhibit a different intestinal microbiota compared to healthy controls, which is enriched in bacterial species from oral origin and microbial peptides derived from these species are pro-inflammatory [54]. In addition, a TLR7-dependent lupus-prone murine model exhibited increased translocation of *Lactobacillus reuteri* to the mesenteric lymph nodes (mLN), liver and spleen and exacerbates lupus-related pathogenesis [55]. Both microbiota-depleted and germ-free mice in the same model displayed reduced tissue damage and pathology, indicating that the microbiota may play a pathogenic role in the onset of SLE. In this line, it is hypothesized that gut microbial dysbiosis can cause damage to the gut barrier, triggering a leaky gut and the susceptibility to develop autoimmune diseases associated with the translocation of bacteria into the circulatory system [56, 57]. In an MRL/lpr model using a mixture of 5 *Lactobacillus* strains as treatment, it was possible to identify a recovery of the renal function in SLE mice. This role was attributed to the increased secretion of IL-10 and its modulation of the T regulatory over Th17 response, which impacts the generation of immune deposits by circulating IgG2a. The positive effect was mainly associated with *L. reuteri* and *Lactobacillus* sp. bacteria from the mixture. Interestingly, this positive effect was identified only in female and castrated male mice but not in non-castrated males, suggesting that these beneficial effects could be related to a sex hormone-dependent manner [58].

Some factors related to Lupus and the permeability of the intestinal barrier are related to immunological components, within which the activation of the NF-κB pathway is essential [59]. In this sense, it has been described that proinflammatory cytokines such as TNF-α, IL-1β, and interferon (IFN)-γ, commonly secreted during an active SLE, can impair the tight junctions’ interactions, affecting the expression of tight junction proteins and their arrangement. Consequently, a negative effect on the modulation of the actin cytoskeleton arrangement in intestinal epithelial cells triggers damage to the gut barrier [60, 61]. The NF-κB activation regulates and maintains the immune homeostasis in gut epithelial tissues, promoting increased apoptosis of the enterocytes in the gut epithelial when this pathway is absent or inhibited [51, 62].

On the other hand, some reports showed that active SLE patients presented minor diverse gut microbiota compared to inactive SLE patients. Interestingly, in the active SLE patients, an increase in the proportion of Proteobacteria has been found [63]. Additionally, a positive correlation between the SLE and the presence of *Streptococcus*, *Campylobacter*, and *Veillonella* bacteria has been identified [64, 65]. Interestingly, a low number of Firmicutes has been observed in SLE patients showing a lower ratio of *Firmicutes* than *Bacteroidetes* [66]. These results are consistent with a study performed in China, where feces and saliva samples from health and SLE patients were evaluated. In this line, the results confirm that a deficient diversity and number of bacteria found in SLE patients’ feces correlate with gut microbiome dysbiosis. However, an increase in this diversity was found in the saliva samples from these patients, with is correlated with the studies of Oral Systemic Lupus (OSL) [67].

*Ruminococcus gnavus* (*R. gnavus*) has been identified in patients with an active SLE compared to healthy control, suggesting that the abundance of this bacteria can be related to SLE disease activity [68]. In contrast, in a B6SKG mice model, it was identified that an increase in the proliferation of segmented filamentous bacteria (SFB) belonging to *Firmicutes* triggered a lupus-like phenotype associated with a high differentiation of T helper 17 (Th17) cells [69]. Furthermore, a murine model of SLE (MRL/lpr) shows a decrease in the number of *Lactobacilli* and an increase in *Lachnospiraceae* improved
disease outcomes in this model [70]. Interestingly, an increase in the abundance of *Lactobacillaceae* has been negatively associated with SLE in mice, suggesting that the administration of these bacteria could be used as a treatment to decrease the risk of triggering SLE [70, 71]. In the (NZW × BXSB) F1 lupus model, an increase in the abundance of *Enterococcus gallinarum* promotes an impairment in the intestinal barrier. Interestingly, *E. gallinarum* can translocate towards the liver of these mice, similar to those found in patients with SLE [72].

Additionally, some compounds from the bacteria, such as lipopolysaccharide (LPS), and from fungi such as (1 → 3)-β-D-glucan molecules, have been identified in sera samples from SLE patients, reinforcing the idea that microorganisms could correlate with SLE disease [73–75]. Both components can stimulate the immune system through the Toll-like receptor type 4 (TLR-4) and dectin-1. This activation promotes the induction of proinflammatory cytokines like type I IFN, which is essential for the activation of B cells for the induction of autoantibodies [76, 77].

On the other hand, it has been reported that a high-salt diet (HSD) might be correlated with gut microbiota dysbiosis and autoimmune progression. This phenomenon can be explained because HSD might activate DCs, modulating the activation of the p38/MAPK-STAT1 signaling pathway and triggering the activation of T Helper 17 (Th17) cells [78]. In line with this, HSD induces accelerated progression of lupus in a murine model by promoting DC activation although its association with increased Th17 activation remains unclear [79].

Another component that can disrupt and weaken the intestinal barrier function is Calprotectin. This molecule is a calcium-containing protein secreted in high yield by macrophages and neutrophils [80]. In SLE patients, this molecule has been identified in stool samples suggesting a weakening function of the impaired intestinal barrier [68, 80]. Also, other factors such as LPS, CD14, and α1-acid glycoprotein were found in serum from SLE patients suggesting the presence of these molecules could be identified by the translocation of bacteria through the intestinal barrier in these patients (Table 1.1) [73, 74, 81–83].

### 1.3.2 Initiation of SLE at the Skin Barrier

A study in SLE patients concluded that 70% of the recruited participants presented oral lesions. The buccal mucosal and lips were the most prevalent zones with damage [48]. Another study performed in the Shahid Mohammadi Hospital in Bandar Abbas showed similar results to those reported by Kudsi et al. [48]. The vast majority of oral lesions were associated with women compared to men. In addition, the main type of lesion identified were mouth ulcers located mainly on the lips and buccal mucosa [84]. In this line, in another study, it was reported that people with active SLE had increased susceptibility to developing problems such as dental caries, periodontitis, and oral mucosal lesions as compared to people with inactive SLE [85]. Initiation of SLE at the skin barrier is not well documented but a recent study reveals that the skin microbiota can contribute to the progression of the disease. The skin colonizer *Staphylococcus aureus* (*S. aureus*) has been considered a fundamental microorganism for developing skin lesions in a mouse animal model characterized by epithelial cell-specific IκBζ-deficient (NfkbizΔK5), observing the induction of different types of autoantibodies and glomerulonephritis with IgG deposition, and increasing the induction of SLE in these animals [86]. In addition, the skin colonization with *S. aureus* promoted increased neutrophil activation and neutrophil extracellular release, which in turn induce the IL-23/IL-17A pro-inflammatory pathway [86]. *S. aureus* has also been reported in active lesions of cutaneous SLE, which colonization is favored by type 1 IFN-mediated barrier disruption [87]. Thus, *S. aureus* and skin dysbiosis can contribute to the initiation and/or progression of SLE (Table 1.1).
1.4 Rheumatoid Arthritis and Its Origin at Mucosal Barriers

Rheumatoid arthritis (RA) is a chronic autoimmune disease which primarily affects the synovial joints, causing pain and disability, but also implies systemic complications, such as cardiovascular diseases [88]. The worldwide prevalence of RA is estimated as 0.51%, being three times higher in women than in men [89, 90]. The immunopathogenesis of RA is driven by autoreactive CD4+ T cells, which activate tissue-destructive functions in macrophages and synovial fibroblasts and promote the production of autoantibodies, that can form immune complexes in the joint [91]. Autoantibodies against IgG (rheumatoid factor, RF) or anti-citrullinated protein antibodies (ACPA) are important diagnostic hallmarks of seropositive RA, although they are only present in 50–70% of RA patients.

Citrullination of autoantigens, catalyzed by the enzyme peptidyl-arginine deiminase (PAD), is a crucial process involved in the immunopathogenesis of seropositive RA [92]. This post-translational modification converts the positively charged amino acid arginine to neutral citrulline and thereby increases the affinity of peptide antigens to the “shared epitope”, expressed by certain RA-associated HLA class II genes [93, 94]. Besides predisposing genetic factors, such as the HLA-DRB1 “shared epitope” and PTPN22 single-nucleotide polymorphism, it has been proposed that environmental triggers at mucosal surfaces might provide important “hits” either by inducing systemic inflammation or promoting the transition from systemic autoimmunity to joint inflammation in RA [92, 95].

The hypothesis of a mucosal origin of RA is supported by the findings that the onset of clinical apparent RA is preceded by the presence in serum of ACPA and RF autoantibodies of the IgA isotype, produced at mucosal surfaces and an increase of circulating IgA+ plasmablasts in at-risk individuals [96, 97]. Furthermore, elevated levels of autoantibodies complexed with the secretory component, necessary for the transport of IgA across the mucosal surface, have been observed in the peripheral blood of patients with early RA [98, 99].

1.4.1 Initiation of Rheumatoid Arthritis by Inflammation and Antigen Citrullination in the Oral Cavity

The oral cavity is considered as a site of first encounter to environmental substances before they enter the gastrointestinal tract and airways [100]. In addition to its permanent contact with microorganisms, food antigens and allergens, the oral mucosa is constantly exposed to mechanical challenges. The oral mucosal barrier consists of a three to five layers of squamous epithelium, contains both keratinized and non-keratinized parts and supplements its chemical and biological defense with saliva [101]. An area of the oral barrier which is particularly vulnerable to microdamage from mastication and colonization by oral pathogens is the tooth-associated mucosa or gingiva [102]. Leakage in the oral mucosa occurs mainly at the inner lining of the gingiva, where the crevicular and junctional epithelium are connected to the tooth surface only through hemidesmosomes and therefore constitute a primary portal for microbial translocation into blood circulation [101]. Even at steady state conditions, the oral mucosa contains a higher proportion of neutrophils than other mucosal barriers and is characterized by an environment that promotes neutrophil recruitment [102]. Neutrophils are the first population of leukocytes that arrive at the site of infection and their principal functions include the recognition, phagocytosis, and degradation of pathogens, as well as the release of granules, and formation of neutrophil extracellular traps (NETs) to immobilize pathogens and prevent their dissemination. NET formation, or NETosis, is a process during which neutrophils release their chromatin complexed with nuclear and cytoplasmic proteins, including PAD [103]. Besides its beneficial effect in eliminating pathogens, the release of NETs also causes damage of host tissue, exposes intracellular
autoantigens, and exacerbates inflammatory responses [104]. It has been shown that local tissue damage induces IL-6 secretion by epithelial cells which promoted protective barrier and antimicrobial responses mediated by IL-17-producing Th17 cells [105]. In contrast, oral pathogens induced aberrant Th17 responses driven by IL-23 and IL-6, and neutrophil activation, conducing to periodontitis with consequent bone resorption and tooth loss [106].

Individuals with RA have a higher incidence of periodontal disease, associated with dysbiotic oral microbiota, while RA symptoms have been shown to improve after periodontitis treatment [107–109]. A study performed in Brazil reported that the subgingival microbiota in RA patients, even in those without periodontitis, was characterized by a higher bacterial load, increased microbial diversity and an enrichment of periodontitis-related bacteria such as Prevotella species, as well as other pathogenic species including Selenomonas spp. and Anaeroglobus geminatus [109]. Interestingly, the presence and abundance of A. geminatus has been shown to correlate with the presence of ACPAs/RF autoantibodies [107]. Oral dysbiosis in RA is associated with elevated levels of inflammatory cytokines IL-2, IL-6, IFN-γ, TNF-α, IL-33, and IL-17 in saliva, compared to healthy subjects [109], which are known promtors of neutrophil recruitment and activation and Th1/Th17 cell differentiation. Accordingly, RA patients showed increased NET formation in circulating and synovial neutrophils compared to healthy and osteoarthritis controls, which correlated with the presence of ACPA and levels of systemic inflammatory markers [104].

An important link between periodontitis and RA is provided by the finding, that the oral microbiome of RA patients is also enriched in citrulline-producing inflammophilic microorganisms, including Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans, keystone pathobionts of periodontal disease, which have been proposed to be directly implicated in the breakdown of immune tolerance to citrullinated antigen epitopes (Table 1.1) [110–114]. In this context, the citrullinome of the periodontal pocket in periodontitis patients was shown to be similar to the spectrum of citrullinated proteins found in the RA joint, including major targets of RA-specific autoantibodies such as citrullinated α-enolase, hnRNP A2/B1 and vimentin [110]. Importantly, dysbiosis of the subgingival microbiota, together with an increase of P. gingivalis, precedes the onset of joint inflammation in individuals at-risk of developing RA, suggesting an important role in the initiation of autoimmune responses [114]. P. gingivalis is unique in its expression of arginine-gingipains and particularly PAD, capable of generating citrullinated host peptides [111], that can be recognized by lymphocytes as neoantigens and might induce the production of ACPA. Another bacterial trigger of oral inflammation, and potentially also RA, is A. actinomycetemcomitans, whose pore-forming virulence factor leukotoxin-A activates endogenous PAD in host neutrophils and the release of hypercitrullinated antigens [110]. However, the association between exposure to P. gingivalis or A. actinomycetemcomitans and the presence of ACPA in RA patients remains controversial [115, 116]. A recent study suggests that immune responses to P. gingivalis-derived citrullinated antigens might contribute to the generation of ACPA in RA patients through molecular mimicry [117, 118].

Although periodontitis alone is probably not sufficient for the development of RA, the characteristic neutrophil and Th17-dominated immune responses at the oral mucosa, which are exacerbated by microbial dysbiosis and the presence of oral pathobionts, might provoke autoantigen citrullination and ACPA production in genetically predisposed individuals, driving systemic and joint-specific autoimmunity.

1.4.2 Initiation of Rheumatoid Arthritis by Antigen Citrullination at the Lung Mucosa

The lung mucosa has been considered as a major site of protein citrullination and initiation of ACPA-positive RA, promoted by infection or
microbial dysbiosis and the inhalation of pollutants such as tobacco smoke [119]. The presence of ACPA in sputum and bronchoalveolar fluid is an early sign of RA [120–122].

Pulmonary manifestations, such as bronchiectasis (defined as irreversible bronchial damage and dilatation) and interstitial lung disease (ILD) are the most common extra-articular manifestations of RA, occurring in almost 70% of patients, but may also be the first signs of RA, preceding articular symptoms [123, 124]. RA with extra-articular manifestation of ILD is associated with IgA RF and secretory component ACPA [125]. The development of RA-ILD is related to a promoter polymorphism in the MUC5B gene that provokes an increased expression of mucin 5B, a highly glycosylated mucus-forming protein secreted by cells in the airway mucosa [126]. Mucin 5B overexpression in the small airways might reduce the mucociliary function which leads to retention of inhaled particles causing more harmful effects including lung injury in case of exposure to pollutants and smoking [2].

Smoking is associated with the presence of IgA ACPA in serum and increases the risk of developing RA in humanized mice and humans expressing the shared epitope, by triggering PAD expression and antigen citrullination in the lung [99, 119, 127]. Citrullination is related to enhanced NET formation by neutrophils and macrophages. It has been shown that subjects at risk of developing RA display increased levels of NET remnants in sputum [128]. NETs contain RA-related citrullinated autoantigens that might induce the generation of ACPA [129]. Due to a loss of integrity of the lung mucosal barrier, e.g., as a product of infection and exacerbated inflammatory responses, ACPA may enter systemic circulation and reach the joints. This hypothesis was strengthened by a proteomic approach of Ytterberg and colleagues, who identified identical citrullinated peptide antigens (derived from citrullinated vimentin) in bronchial biopsies and synovial tissues specimen of RA patients, supporting the link between antigen-citrullination in the lung and joint inflammation [130].

It has been reported that smoking can also induce alterations of the lung microbiota [131], although this has not been studied so far in the context of RA. Scher and colleagues analyzed the microbial composition of bronchoalveolar lavage fluid (BAL) of patients with early untreated RA, sarcoidosis and healthy controls by 16S rRNA gene sequencing and found distal airway dysbiosis in both patient groups, with an overrepresentation of Pseudonocardia, characteristic to RA patients [116]. At the lung mucosa, inhalation of tobacco smoke or other pollutants, epithelial damage, microbial infections or dysbiosis might act either separately or together to induce local inflammation, associated with the release of NETs and autoantigen citrullination, leading to ACPA production and translocation into systemic circulation in individuals genetically predisposed to develop ILD and RA (Table 1.1).

1.4.3 Dysbiosis of Intestinal Microbiota and “Leaky Gut” as Triggers of Inflammation in Rheumatoid Arthritis

Current research focuses particularly on the role of the intestinal barrier integrity in the development of RA [132]. In the gut, the physical barrier is formed by a layer of epithelial cells, closely connected by tight junction proteins that regulate the paracellular pathway. The overlaying chemical barrier is formed by the mucus layer, limiting direct interactions with microbes and large molecules [1]. The breakdown of the intestinal barrier, e.g., by apoptosis of intestinal epithelial cells due to microbial infection, creates a proinflammatory environment that promotes the differentiation of autoreactive Th17 cells [133]. Pro-inflammatory cytokines, particularly IFN-γ and TNF-α, promote dysfunction of the epithelial barrier by causing ZO-1 internalization, occluding downregulation, and apoptosis in epithelial cells [134, 135]. The permeability of the gut epithelium to small molecules is regulated by zonulin, a precursor of haptoglobin-2, which
signals through co-binding of protease-activated receptor 2 and epidermal growth factor receptor, resulting in a protein-kinase-C-dependent disassembly of the tight junction complex [136]. Zonulin is secreted by intestinal epithelial cells in response to dietary stimuli, such as gluten, or enterobacteria [137]. Recent studies by the groups of Zaiss and Mauri independently revealed a critical role of the intestinal barrier integrity and particularly zonulin, in the transition from asymptomatic systemic autoimmunity to inflammatory joint disease in RA [138, 139]. Levels of zonulin were shown to be increased in serum of RA patients, including individuals with pre-clinical RA, while the expression of tight junction proteins occluding, claudin-1, and zonula occludens-1 was decreased in the intestinal epithelium of RA patients as compared to healthy controls [138, 140]. Accordingly, bacterial LPS and gut permeability markers such as LPS-binding protein (LBP) and soluble CD14 (sCD14) were shown to be increased in serum of individuals with pre-clinical and early RA, indicating that a leaky barrier and the translocation of microbial components precede the development of articular symptoms [138, 139]. In established RA, the increased serum levels of LBP and soluble sCD14 correlated with an increased disease activity [140].

Enhanced gut permeability and intestinal inflammation were also shown to precede the onset of arthritis in a mouse model of collagen-induced arthritis [138]. Arthritis has been demonstrated to be accompanied by an increased migration of immune cells to the intestinal mucosa in humans and mice, indicated by elevated proportions of cells expressing the gut homing chemokine receptor CCR9 [139]. Interestingly, both dysbiotic microbiota and pro-inflammatory leukocytes were necessary to disrupt gut barrier integrity [139]. Matei and colleagues also demonstrated that arthritis-associated microbiota and inflammatory cytokines like IFN-γ diminish IL-10 receptor expression by intraepithelial lymphocytes, which further contributes to the disruption of gut barrier integrity and joint inflammation [139]. Restoration of the epithelial barrier function by the administration of a zonulin antagonist ameliorated experimental arthritis in mice and might therefore be a promising therapeutic target for the treatment of RA [138, 139]. The presence and abundance of tight-junction-disrupting bacteria, such as Collinsella aerofaciens and P. gingivalis in feces of RA patients supports a role of these bacteria in enhancing gut permeability [141, 142].

Cheng and coworkers performed fecal metagenomic studies on four stages of RA and found stage-specific alterations of microbial species during disease progression [143]. C. aerofaciens was found significantly enriched only in the first stage of RA, indicating its contribution to the early breach in gut barrier integrity. This is supported by a previous report, demonstrating the ability of C. aerofaciens to increase epithelial permeability and IL-17 expression in vitro and to induce severe arthritis in the collagen-induced arthritis (CIA) model [141]. Using the K/BxN mouse model of autoimmune arthritis, Teng and colleagues demonstrated that gut commensal segmented filamentous bacteria (SFB) are able to drive the differentiation of T follicular helper (Tfh) cells in Peyer’s patches, by inhibiting IL-2 receptor signals, and to promote the migration of these Tfh cells from gut to systemic sites where they can induce the production of autoantibodies [144].

Immune responses at the gut mucosa are regulated by metabolites of commensal bacteria, such as short chain fatty acids (SCFA) and an immunoregulatory environment that favors the generation of T<sub>reg</sub> cells [145]. SCFA are produced by the fermentation of dietary fibers and exert protective effects on the intestinal epithelial barrier and the host immune system. It has been shown that SCFA are also able to suppress inflammatory arthritis in mice [146]. Microbial dysbiosis is known to affect intestinal SCFA levels [147]. Accordingly, a recent study by Rosser and colleagues reported reduced levels of the SCFAs butyrate and propionate in feces of RA patients and an increase in serum acetate compared to healthy controls, indicating a perturbation of microbiota composition [148]. A high-fiber diet has been shown to favor
SCFA-producing bacteria, which had beneficial effects on the intestinal barrier and reduced disease activity in RA patients [149].

Comparison of the fecal microbiome of transgenic mice expressing the RA-susceptible HLA-DRB1*0401 transgene with those expressing RA-resistant HLA-DRB1*0402 revealed an increase in Clostridium-like bacteria in susceptible mice, related to an increased intestinal permeability and inflammatory Th1/Th17 gene transcripts in the small intestine [150]. Studies of the human microbiome demonstrated concordance between oral and intestinal microbiomes as well as microbial dysbiosis in RA patients, with RA-specific overrepresentation of Ligilactobacillus salivar ius, C. aerofaciens and Prevotella copri, dependent on the analyzed geographic population [141, 150, 151]. Prevotella species, and particularly P. copri, were enriched in a proportion of individuals with pre-clinical seropositive and recent-onset untreated RA [151, 152].

Pianta and colleagues described Th1 responses to P. copri peptides in the peripheral blood of 42% of patients with new-onset RA, as well as IgG and IgA antibodies to the P. copri protein of origin Pc-p27 in 16% and 10% of all RA patients, respectively [153]. The same group also identified two immunogenic synovial autoantigens, N-acetylglucosamine-6-sulfatase and filamin A, which share sequence homology with Prevotella and trigger lymphocyte responses in 35% and 42% of RA patients, respectively, suggesting that molecular mimicry between microbial and self-peptides might contribute to autoimmune responses [153]. P. copri can overgrow commensal intestinal microbiota and promotes Th17 cell responses in the large intestine, by inducing the expression of Th17-driving cytokines IL-6 and IL-23 in antigen-presenting cells [151, 154]. In the zymosan-induced SKG mouse model of arthritis, administration of RA-derived microbiota enriched with Prevotellaceae, or P. copri alone, promoted a more severe arthritis [154]. A current study demonstrated that P. copri decreases the thickness of the intestinal mucus layer, pointing to its contribution to the impaired intestinal barrier function observed in RA [155].

Only recently, Chriswell and coworkers identified an arthritogenic strain of Subdoligranulum in feces isolates, by binding of monoclonal IgG and IgA autoantibodies. This strain of the Lachnospiraceae/Ruminococcaceae genus stimulated in germ-free mice the expansion of Th17 cells, production of RA–associated IgG autoantibodies and joint swelling [156]. Taken together, loss of barrier integrity at the intestinal mucosa, induced by dysbiotic microbiota, overgrowth of certain bacterial species and subclinical gut inflammation mediated by Th17 cells, favours the translocation of microbial components and the induction of systemic inflammation promoting autoimmunity and articular manifestations of RA (Table 1.1).

### 1.5 Conclusions

Although the systemic manifestations of food allergy, SLE and RA are quite different, the epithelial and mucosal barriers represent important sites that can contribute to the initiation and/or progression of these autoimmune diseases (Fig. 1.1). The microbiota inhabiting the skin, the intestine, lung, and the oral cavity can be altered following infection or damage leading to dysbiosis. As discussed above, some bacterial species and genus may be either beneficial or pathogenic by releasing different molecules that can modulate immune responses towards self-antigens and dietary antigens. Strengthening of the beneficial properties of gut commensals may help to develop effective therapies against autoimmune diseases but its use is still under evaluation. Dysbiosis may also be associated with increased barrier permeability, which in turn may contribute to increased antigen passage to the circulation and increase the chances of developing exacerbated autoimmune responses. It is unclear whether other environmental factors may contribute to barrier permeability in the autoimmune diseases discussed in this review and further research is required to elucidate the
mechanisms that may modulate barrier function in autoimmunity.

Several lines of research are investigating the role of different epithelial- and immune cell-derived signals present at host barriers that promote systemic autoimmunity in the autoimmune diseases discussed in this manuscript (Table 1.1). In food allergy, there is extensive research about the role of innate and adaptive cells associated with type 2 responses residing in the skin and the intestinal mucosa, including ILC2, basophils, mast cells and Th2 cells, which may support the generation of allergen-specific IgE and the potential induction of anaphylactic responses. In contrast, initiation and progression of RA has been associated with NET formation, antigen citrullination and the generation of autoreactive Th17 cells, which may be generated at the oral cavity and/or the intestine and may migrate systemically. In the case of SLE, although there is evidence of the importance of the skin and intestinal barriers in the pathogenesis of the

Fig. 1.1 Pathogenesis of autoimmune diseases at different host barriers. Systemic lupus erythematosus: SLE pathogenesis can involve the oral cavity, exhibiting deposits of IgG, IgA, and IgM immunoglobulins and the C3 protein; the skin, where S. aureus can induce neutrophils and the IL-23/IL-17 axis promoting systemic disease and the intestine where gut dysbiosis can alter the T<sub>reg</sub>/Th17 balance and contribute to disease pathogenesis. These local responses contribute to the systemic presence of autoreactive T cells and autoantibodies against ANAs and smith. Rheumatoid arthritis: RA pathogenesis can involve the oral cavity, where oral bacterial pathogens and neutrophils can contribute to tissue damage and later to antigen citrullination; the intestine, where dysbiosis can promote altered T<sub>reg</sub>/Th17 balance and pathogenic autoreactive responses and the lung, where environmental triggers such as smoking can promote neutrophil pathogenic function leading to antigen citrullination. These local responses contribute to the systemic presence of autoreactive Th1/Th17 cells and autoantibodies against RF and ACPA. Food allergy: food allergy pathogenesis can involve the skin, where tissue damage and antigen sensitization contribute to the release of inflammatory signals and increase in ILC2 and the systemic release of these signals can promote food allergy in the intestine. Intestinal inflammation can be promoted in response to skin allergen sensitization or independently by the increase in ILC2, mast cells, basophils and Th2 responses, contributing to systemic responses. These local responses contribute to the systemic presence of allergen-specific IgE, which could induce anaphylactic reactions. This figure was created with BioRender.com.
disease, much less is understood about the immune mechanisms regulating its initiation and progression at those barriers.

**Statements and Declarations**

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Statins and Hemostasis: Therapeutic Potential Based on Clinical Evidence

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Abstract

Hemostasis preserves blood fluidity and prevents its loss after vessel injury. The maintenance of blood fluidity requires a delicate balance between pro-coagulant and fibrinolytic status. Endothelial cells (ECs) in the inner face of blood vessels maintain hemostasis through balancing anti-thrombotic and pro-fibrinolytic activities. Dyslipidemias are linked to hemostatic alterations. Thus, it is necessary a better understanding of the underlying mechanisms linking hemostasis with dyslipidemia. Statins are drugs that decrease cholesterol levels in the blood and are the gold standard for treating hyperlipidemias. Statins can be classified into natural and synthetic molecules, approved for the treatment of hypercholesterolemia. The classical mechanism of action of statins is by competitive inhibition of a key enzyme in the synthesis pathway of cholesterol, the HMG-CoA reductase. Statins are frequently administrated by oral ingestion and its interaction with other drugs and food supplements is associated with altered bioavailability. In this review we deeply discuss the actions of statins beyond the control of dyslipidemias, focusing on the actions in thrombotic modulation, vascular and cardiovascular-related diseases, metabolic diseases including metabolic syndrome, diabetes, hyperlipidemia, and hypertension, and chronic diseases such as cancer, chronic obstructive pulmonary disease, and chronic kidney disease. Furthermore, we were prompted to delved deeper in the molecular mechanisms by means statins regulate coagulation acting on liver, platelets, and endothelium. Clinical evidence show that statins are effective regulators of dyslipidemia with a high impact in hemostasis regulation and its deleterious consequences. However, studies are required to elucidate its underlying molecular mechanism and improving their therapeutic actions.
2.1 Introduction

Hemostasis is a complex system in which vascular cells, blood cells and circulating coagulation factors, interact to preserve blood fluidity and prevent its loss after vessel injury [1]. The maintenance of blood fluidity requires a delicate balance between pro-coagulant and fibrinolytic status [2] and the loss of equilibrium, usually concludes in thrombotic or hemorrhagic episodes [3].

Endothelial cells (ECs) located in the lumen of blood vessels play a critical role in hemostasis maintenance by balancing anti-thrombotic and pro-fibrinolytic activities [4] (characterized by the release of nitric oxide (NO), antithrombin (AT), protein C, protein S, tissue factor pathway inhibitor (TFPI) and tissue-type plasminogen activator (tPA) [5]) with pro-thrombotic and anti-fibrinolytic activities (characterized by the release of von Willebrand Factor (vWF), plasminogen activator inhibitor (PAI-1), thrombin activable fibrinolysis inhibitor (TAFI), selectins, intercellular adhesion molecule 1 and 2 (ICAM-1, ICAM-2) and vascular cell adhesion molecule 1 (VCAM-1) [6, 7]). Similarly, circulating cells, such as platelets, monocytes, and leukocytes, play an important role in thrombosis due their expression of pro-thrombotic molecules as tissue factor (TF), phosphatidyl serine (PS), glycoprotein IIIa (GPIIIa) and selectins [8], as well as their capacity to recruit more of these cells to adhere and form stable clots [9]. Furthermore, the liver is pivotal in maintaining the balance of
hemostasis by synthetizing all blood clotting factors, including pro-thrombin (PT) and fibrinogen [10]. When the balance of hemostasis favors thrombosis, biomarkers of thrombosis progression (e.g., fibrin and D-dimer) are generated, and their quantification offers an objective measure for patient diagnosis and prognosis [11].

The onset of pro-coagulant states is a marker of severity, in viral diseases such as hepatitis B, and the levels of AT, which critically fell in patients with severe disease, correlated with hospitalization length [12]. Furthermore, in patients diagnosed with chronic hepatitis B and C, a decrease in plasma AT was associated with hepatocellular damage and liver disease progression. Interestingly, a reduction in the anticoagulant protein C was also detected in critically ill patients and showed better sensitivity as marker to discriminate liver disease stages [13]. Similarly, in HIV patients, a reduction in protein C levels, and particularly its cofactor, protein S, was detected and linked to an increased thrombotic risk [14]. Similar findings have been described in neonatal septic patients [15] as well as patients with biliary acute pancreatitis [16].

In ulcerative colitis, which is characterized by hypercoagulable states and microthrombus formation, serum levels of TFPI (inhibitor of extrinsic coagulation pathway) increased significantly, suggesting a relevant participation in the pathogenesis and progression of the disease [17]. TFPI levels also demonstrated adequate sensitivity, specificity and positive predictive value in the diagnosis of asymptomatic venous thromboembolism (VTE) in patients with epithelial ovarian cancer [18]. Similar findings were described in patients with concomitant lung cancer for the diagnosis of deep venous thrombosis (DVT) and tumor metastasis [19].

The participation of adhesion proteins in thrombosis-associated diseases such as coronavirus disease 2019 (COVID-19) has also been subject of analysis. vWF, for example, increased in COVID-19 patients and related to disease severity [20] and mortality [21]. Selectin levels at admission of COVID-19 patients exhibited a considerable increase, especially in those experiencing thrombosis during hospitalization. Remarkably, P-selectin, E-selectin and L-selectin were predictors for thrombosis with 97.1, 97.6 and 96.5% sensitivity, while the three exhibited a 100% [22]. ICAM-1 expression was significantly enhanced in COVID-19 patients [23] and VCAM-1 was increased in lung samples and circulation from death COVID-19 patients, suggesting a local and systemic contribution of adhesion molecules in thrombotic disbalance [24].

The interplay between circulating cells and plasma coagulation factors is well recognized in the etiology of thrombosis [25]. Platelet activation has been linked to hypercoagulation in type-2 diabetes [26], platelet-monocyte aggregates and subsequent increase in tissue factor expression was observed in patients with severe COVID-19 [27], enhanced platelet–eosinophil interactions have been linked to thrombosis in atherosclerosis [27] and neutrophils and neutrophil extracellular traps are structural components of ischemic stroke thrombi [28]. Thrombin activation and thrombin fragments F1 + 2 shown to be considerably higher in patients with peripheral artery disease and chronic limb ischemia and were associated to exacerbated hypercoagulability and thrombotic risk [29]. Fibrinogen, the precursor of fibrin (the network-forming strands that trap circulating cells and supports clot structure) [30] demonstrated a significant decrease in postpartum hemorrhage [31], a significant increase in patients with inflammatory bowel disease, enhancing hypercoagulable status [32], a higher correlation with mortality in cancer patients [33], and a strong predictive value in early acute coronary syndrome diagnosis [34].

The primary fibrinolytic protein, tPA, has a highly affinity to fibrin [35], which explain its increased reactivity in thrombotic events [36]. tPA was shown to have a considerable rise and strong connection with poor respiratory function and death in COVID-19 patients [37]. Similarly, demonstrated enhanced activity in type-2 diabetic patients, acting as an early predictor of lower extremity arterial disease [38] and exhibited increased expression in patients with acute
pulmonary embolism (PE), demonstrating a high sensitivity and negative predictive value to exclude PE [39].

tPA is essential for fibrin breakdown and thrombus resolution since it catalyzes plasminogen conversion into plasmin [40]. The generation of fibrin degradation products, as D-dimer offers an indirect measure of plasmin activity and fibrinolysis progression. Despite its low specificity, a rise in plasma D-dimer is an unequivocal sign of local or systemic coagulation activation [41]. D-dimer has been extensively studied and its increase is a reliable marker for the diagnosis of life-threatening conditions as disseminated intravascular coagulation in sepsis [42], DVT [43], VTE after lower limb fractures surgery [44], hypercoagulability and preeclampsia severity [45], severity of acute superior mesenteric venous thrombosis [46], severity of acute pancreatitis [47], severity [48] and in hospital mortality of COVID-19 patients [49].

Dyslipidemias, or disorders of the lipid profile [50], are highly prevalent worldwide and have been strongly associated with life-threatening complications in patients with cardiovascular disease (CVD) [50], diabetes [51], kidney disease [52] and liver disease [53]. Interestingly, dyslipidemias have been linked to hemostatic alterations [54]. While low-density lipoproteins (LDLs), one of the main types of circulating lipoproteins, are widely recognized for their atherosclerotic and atherothrombotic properties [55], high-density lipoproteins (HDLS) are widely recognized as “good” or “protective” [56]. However, recent evidence refutes the latter by linking HDLs to thrombosis [57, 58] and increased levels of D-dimer [59]. Moreover, the oxidized form of HDL has been associated with increased coagulation and reduced fibrinolysis in type 2 diabetes patients [60] and increased procoagulant phenotype in platelets [61]. In fact, it has been described that HDL, promoted thrombin formation, increasing the risk of hypercoagulability during dyslipidemia, [62]. Moreover, hyperlipidemic adult patients present alterations in thrombotic and fibrinolytic molecules such as vWF, tPA and PAI [63] and, similarly, hyperlipidemic pediatric patients present dysfunctional fibrinolytic activity [64, 65]. Significantly, coagulation-associated complications account for close to 100,000 annual deaths in the United States [66], highlighting how necessary a better understanding of the underlying mechanisms is to pave the way for future therapies.

Statins are drugs that lower cholesterol levels in the blood [67] and are the gold standard for treating hyperlipidemias [68]. Based on their origin, statins can be classified into natural and synthetic molecules (Fig. 2.1). Natural statins include lovastatin, simvastatin and pravastatin, all deriving from fungi and sharing a similar structure [69]. Lovastatin is isolated from Aspergillus terreus and is fully approved for the treatment of heart disease and hypercholesterolemia [70]. The active metabolite of lovastatin, beta-hydroxy acid, is produced in the stomach after lovastatin hydrolysis [71]. Simvastatin is a semi-synthetic statin derived from lovastatin [72], and like its precursor, it is a prodrug used in the inactive lactone form, with beta-hydroxy acid also as the active metabolite [73]. Conversely, pravastatin is derived from mevastatin, a statin isolated from Penicillium citrinium [74], further biotransformed by Streptomyces carbophilus [75], which is administered in its active form [76]. Synthetic statins include atorvastatin, rosuvastatin, pitavastatin, fluvastatin, and cerivastatin, all of which share fluorophenyl groups in their structure [69]. Atorvastatin is the most frequently administered statin for the treatment of hypercholesterolemia and is used as a calcium salt of hydroxy acid, its active form, in a posology ranging from 10 to 80 mg a day [77]. Rosuvastatin, outstanding by its hepatic specificity [78], is considered the most potent statin and the most effective in the reduction of circulating cholesterol [79]. It is administered as calcium salt in a posology ranging from 5 to 40 mg per day [80]. Pitavastatin, also named nisvastatin [81], is a novel statin [82] with a maximal approved dosage (4 mg/day) showing an efficiency lowering cholesterol levels equivalent to simvastatin/atorvastatin and superior to pravastatin [83]. Fluvastatin, the carboxylic acid, is a statin highly permeable in the intestine [84] which action in
lowering cholesterol levels is attributed to the increase in expression of LDL receptor in hepatocytes and LDL catabolism [85]. Finally, cerivastatin, was recalled due strong secondary effects, especially rhabdomyolysis with subsequent death [86].

Based on their solubility profile, statins are classified as hydrophilic (rosuvastatin and pravastatin, with great hepatoselectivity [87] and action mediated by specific carriers [79]), or lipophilic (simvastatin, atorvastatin, cerivastatin, fluvastatin, pitavastatin and lovastatin, which are associated with pleiotropic effects [88] due their ability to cross the cell membrane by passive diffusion [89]).

The canonical mechanism of action of statins is by competitive inhibition of HMG-CoA reductase, a key enzyme in the synthesis pathway of cholesterol. Another mechanism is the inhibition of isoprenoids, reducing HMG-CoA to mevalonic acid [90]. Evidence shows that the inhibition of HMG-CoA reductase induces the expression of LDL receptors and the increased presence of this receptors in the plasma membrane increases the uptake of LDL particles from circulation by liver cells [91].

The most frequent administration route of statins is oral and after the uptake, present a highly variable exposure [92]. Atorvastatin and rosuvastatin for instance, both lipophilic, presenting the highest half-life times, with 20 h and 7–20 h respectively, while the rest, exhibited lower times. Pitavastatin in addition, has a half-life of 10 h, fluvastatin 1–3 h, cerivastatin 2–3 h, pravastatin 1–3 h, lovastatin 2–5 h, simvastatin 2–5 h [93]. The bioavailability is also highly variable. Rosuvastatin, exhibited 20% after an oral dose of 40 mg/day (PMID: 14667956).
Atorvastatin 15% [94], lovastatin 5% [95] and simvastatin 5% after a dose of 80 mg/day [96]. In this matter, the design of new drug delivery systems is showing a promising potential, considering the increased circulation time and reduced liver uptake that translate in improved treatment efficiency [97].

Statins are primarily metabolized by cytochrome P450 CYP3A4 isoform, except for fluvastatin that is metabolized by CYP2C9 isoform pathway [79]. Not all statins show the same behavior after administration. Atorvastatin and rosvastatin show a long half-life due to, among other characteristics, their metabolites remain active, prolonging the inhibition of HMG-CoA reductase [98, 99]. In the other hand, due to their short half-life, the other statins must be administered at evening, when cholesterol synthesis increases to take advantage of their effect [90]. Renal excretion of statins is low, encompassing from 5 to 30%. A major part of metabolites is eliminated via bile secretion [79]. The oral administration of statins also involves a strong hepatic first-pass extraction, metabolizing between 70 and 80% of statin administered [92]. But fluvastatin exhibited limited systemic exposure after the extensive sequestration in the first-pass metabolism that implies a brief half-life of 30 min, reduced active metabolites and adverse effects attributed to drug accumulation [100]. In addition, circulating statins are predominantly bound to proteins, reducing even more their bioavailability in the circulation and peripheral tissues [92]. In this context, hepatic diseases could mean a relevant factor to consider before statin administration in comparison to renal insufficiency, depending on the statin used.

Importantly, the action of statins has shown elevated interaction with other drugs. It is known for instance, that simvastatin (metabolized by the enzyme CYP3A4) is affected using CYP3A4 inhibitors as azole antifungals (itraconazole), macrolide antibacterial (erythromycin and clarithromycin), and calcium channel antagonists (diltiazem, mibebradil, and verapamil) that significantly increased simvastatin AUC [101]. Conversely, ferburtinib, a CYP3A4 substrate, increased the AUC of simvastatin about 150% [102], while Amlodipine, a CYP3A4 inhibitor, increased in approximately 80% the AUC of simvastatin [103]. Similarly, atorvastatin is metabolized by CYP3A4, being the AUC also affected by the enzyme inhibitors [101]. Additionally, the inhibition of OATP1B (statins transporter) with a single intravenous injection of rifampicin increased the AUC of atorvastatin by about seven times compared to its baseline levels [104]. Finally, lovastatin reduced AUC and Cmax after 3 days of berberine (an insulin sensibilizing drug) also mediated by CYP 450 3A4 [105].

The use of pravastatin and the OATP inhibitor cyclosporine, showed a significant increase in pravastatin concentrations at plasma levels [106, 107]. Similarly, glecaprevir and pibrentasvir, antiviral drugs against chronic hepatitis C virus infection and inhibitors of OATP1B1 and OATP1B3, increased the AUC of this statin in approximately 130% in healthy subjects [108]. For rosvastatin, the use of darolutamide, a drug approved for the treatment of prostate cancer increased the AUC of rosvastatin to about 400% [109]. In addition, Recent studies found that a single 600 mg dose of rifampin (a strong inducer of CYP3A4) administered either orally or intravenously increased the AUC of rosvastatin by 300% [110, 111].

Pitavastatin co-therapy with lopinavir/ritonavir decrease its AUC by 20% in healthy adult volunteers, showing its potential for the safe treatment of dyslipidemia in patients with HIV and receiving antiretroviral therapy [112]. The co-administration of cyclosporine with pitavastatin generated an increase in the AUC of pitavastatin by about five times its basal AUC [113], and a similar effect that is obtained when co-administering a single dose of rifampicin together with pitavastatin [114]. Fluvastatin with cimetidine, ranitidine, or omeprazole, generated an increase in Cmax of 43, 70 and 50% respectively with each drug, while the AUC increased in a range of 24–33% when fluvastatin was used with these drugs, decreasing plasma clearance between 18 and 23% [115, 116]. In addition, the use of fluvastatin with rifampin reduced fluvastatin AUC by 50% [117].
Finally, the interaction of food supplements with statins also showed remarkable effects. Lovastatin for instance, reduced the exposure of its active metabolite (lovastatin acid) in circulation after a high fat diet and increased the exposure of the inactive form in muscles, promoting myotoxicity by the downregulation of carboxylesterase (CES) activity, an effect prevented by the intake of isoflavones that improve CES activity and subsequently, increase the circulating levels of lovastatin acid [118]. Similarly, has been reported that supplementation with essential amino acids enhance the effect of rosuvastatin and improved cellular energetics and renal function [119]. For simvastatin, it has been described that the supplementation with vitamin D promoted a significant reduction in the bioavailability of its active and inactive forms (simvastatin acid) [120]. Atorvastatin in the other hand, has showed potentiated effect in lowering circulating lipid levels and treat hyperlipidemia when is administered with coenzyme Q10, although the associated mechanisms remain to be clarified it is interesting that this dual treatment also reduced myotoxicity [121]. Similarly, pravastatin showed improved effect when combined with N-3 fatty acid supplementation, diminishing LDL cholesterol particles in hyperlipidemic patients. However, molecular mechanisms are still unexplored [122]. Pitavastatin has showed delayed absorption after a high-fat meal although that did not represent any adverse effect [123], whereas Fluvastatin absorption remained unaffected after eating [100].

2.2 Statins in Thrombotic Events

Beyond its role in the regulation of lipid profile, statins have shown pleiotropic effects including the regulation of inflammatory signaling [124], fibrosis reduction in hepatitis C patients [125], antioxidant properties in the inhibition of superoxide synthesis by macrophages and other reactive oxygen spices (ROS) from endothelium [90]. However, the study of statins effectivity in the treatment of hemostatic (thrombotic/fibrinolytic and hemorrhagic) alterations remain as an unsolved issue, considering the variability observed in clinical trials depending on statin subtype used, administered dose, treatment time and concomitant pathology (Table 2.1).

In randomized controlled studies performed in patients experiencing VTE, rosuvastatin is the most used statin. A study including 247 patients, shows that the 126 patients randomized to rosuvastatin treatment group improved coagulation parameters including reduced circulating levels of coagulation factor VIII (FVIII), vWF, factor XI (FXI), factor VII (FVII) and D-dimer after one month under treatment [126]. Similarly, in a cohort of 228, the group of patients receiving rosuvastatin (10 mg/day) for three months showed decreased levels of D-dimer, suggesting an attenuation in the thrombotic event, together with a visible reduction in mean platelet volume, an indicator of platelet activation [133]. Likewise, a study including a cohort of 245 patients experiencing VTE, where 125 were submitted to rosuvastatin treatment for 4 weeks, exhibits that drug users decreased endogenous thrombin potential [127]. Similarly, the reduction in mean clot lysis time, an indicator of plasma fibrinolytic potential, was observed in a study including 126 patients with previous VTE treated 28 days with rosuvastatin. This treatment also decreased plasmin inhibitor and TAFI expression, and increased fibrinogen expression while plasminogen activator inhibitor I (PAI-I) did not change levels [128]. Likewise, patients with deep venous thrombosis treated with rosuvastatin for 28 days showed improved levels of procoagulant phospholipids activity independent of plasma cholesterol levels [129].

Similarly, a study performed in 818 patients with DVT of which 34% were under stain therapy with atorvastatin (10–80 mg/day), simvastatin (20–80 mg/day) and pravastatin (10–80 mg/day) for 3 months showed that the exposition to statins was associated to improved thrombus resolution in patients with DVT. However, that effect was not associated with reduced thromboembolic recurrence or mortality [165]. Interestingly, the use of rosuvastatin plus heparin in the treatment of DVT for three months did not promote significant changes in the
Table 2.1 Statins effect in hemostatic alterations considering administered dose, study duration, number of participants, primary pathology and results from clinical studies

| Statin                  | Dose mg/day | Duration | n  | Pathologies       | Results                                                                                   | References |
|-------------------------|-------------|----------|----|-------------------|-------------------------------------------------------------------------------------------|------------|
| **Statin in thrombotic events**                                 |
| Rosuvastatin            | 20          | 1 month  | 247| VTE               | Reduced FVIII, vWF, FXI, FVII and D-dimer                                                | [126]      |
| Rosuvastatin            | 20          | 4 weeks  | 126| VTE               | Reduction on endogenous thrombin potential                                               | [127]      |
| Rosuvastatin            | 20          | 28 days  | 126| Previous VTE      | Mean clot lysis time, plasmin inhibitor and TAFI decrease. PAI not change, fibrinogen   |
|                         |             |          |    |                   | increased                                                                                 | [128]      |
| Rosuvastatin            | 20          | 28 days  | 245| Previous VTE      | Reduction of procoagulant phospholipids, not explained by changes in total cholesterol  |
|                         |             |          |    |                   | nor change in levels of total- or platelet-derived EVs                                 | [129]      |
| Atorvastatin            | 10–80       | 3 months | 818| DVT               | Improved thrombus resolution                                                            | [130]      |
| Simvastatin             | 10–80       |          |    |                   |                                                                                          |            |
| Pravastatin             | 10–80       |          |    |                   |                                                                                          |            |
| Rosuvastatin            |             | 3 months | 230| DVT               | No effect on D-dimer                                                                    | [131]      |
| Simvastatin, atorvastatin, fluvastatin, rosuvastatin and pravastatin | NA | 3 months | 156| Suspected pulmonary embolism | Decreased D-dimer                                                                       | [132]      |
| Rosuvastatin            | 10          | 3 months | 228| VTE               | Decreased D-dimer and mean platelet volume                                              | [133]      |
| **Statin in vascular and cardiovascular-related diseases**       |
| Atorvastatin            | 10          | 4 weeks  | 35 | Heart failure     | Anti-thrombin III, protein C, FV, tPA and PAI decreased with atorvastatin. Plasma vWF,  |
|                         |             |          |    |                   | FVII, protein S remain unaffected                                                        | [134]      |
| Atorvastatin            | 10 and 80   | 2 weeks  | 60 | Acute coronary syndrome | Low and high atorvastatin dose suppress elevated expression of vWF | [135]      |
| Atorvastatin            | 10          | 6 weeks  | 45 | Unstable angina   | Atorvastatin prevent increase in antithrombin III and vWF. No variations in tPA protein |
|                         |             |          |    |                   | S, FVIII and protein C                                                                   | [136]      |
| Atorvastatin            | 80          | 8 weeks  | 90 | Arterial occlusive disease | Atorvastatin reduced thrombin generation and TF, GPIIIa and p-selectin of   |
|                         |             |          |    |                   | platelet derived microparticles from patients with peripheral vascular disease          | [137]      |
| Statin        | Dose  | Duration | n  | Pathologies                          | Results                                                                                                                                                                                                 | References |
|--------------|-------|----------|----|--------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Atorvastatin | 20 mg | N/A      | 59 | Carotid atherosclerotic plaque       | TF Ag and TFPI Ag levels, and TF activity in plaques after atorvastatin treatment were lower                                                                                                             | [138]      |
| Rosuvastatin | 40 mg | N/A      | 54 | Acute coronary syndrome              | Reduced interactions between platelet-neutrophils and platelet-monocytes. No effect on platelet aggregation, sP-selectin                                                                                   | [139]      |
| Simvastatin  | 80 mg | 15 days  | 32 | Aneurysmal subarachnoid hemorrhage   | No changes in coagulation, fibrinolysis o endothelial function after statin treatment                                                                                                                  | [140]      |
| Pravastatin  | 20–40 | 6 months | 93 | Coronary artery disease and cholesterolmia | Decreased PAI and tPA antigen. No changes in D-dimer, prothrombin, FVIIa, vWF. Ex-vivo decreased thrombus area in non-CAD. CAD patients decreased thrombus formation | [141]      |
| Fluvastatin  | 80 mg | 6 h      | 57 | Acute coronary syndrome              | Soluble endothelial protein C receptor increased and free TFPI increased after administration of fluvastatin                                                                                             | [142]      |
| Pravastatin  | 40 mg | 3 months | 50 | CVD                                  | Significant reduction in blood thrombogenicity and endothelium-dependent vasoresponse. No effect on fibrinogen, sL-sP-selectin and sICAM-1                                                               | [143]      |
| Simvastatin  | 20 mg |          |    |                                      |                                                                                                                                                                                                       |            |
| Simvastatin  | 40 mg | 4 weeks  | 38 | Coronary artery disease              | Increased plasma clot permeability, short fibrinolysis time, increased clot porosity                                                                                                                  | [144]      |
| Atorvastatin | 40 mg |          |    |                                      |                                                                                                                                                                                                       |            |
| Simvastatin  | 40 mg | At least 1-month prior admission | 19 | Myocardial infarction                | Reduction in thrombin-antithrombin complexes in the vascular injury site, but not in circulation                                                                                                        | [145]      |
| Atorvastatin and simvastatin | 20–40 | 1 year  | 58 | Coronary heart disease               | Increased D-dimer and tPA activity and reduced tPA antigen in atorvastatin group. In simvastatin groups was observed reduced prothrombin 1.2 fragments. No differences in coagulation variables | [146]      |

Metabolic diseases and related risk factors (metabolic syndrome, diabetes, hyperlipidemia, hypertension)

| Statin        | Dose  | Duration | n  | Pathologies         | Results                                                                                                                                                                                                 | References |
|--------------|-------|----------|----|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Atorvastatin | 80 mg | 12 weeks | 88 | Metabolic syndrome  | Improved lipidic profile, no changes in ICAM-1, platelet activity. Increased VCAM-1 activity                                                                                                           | [147]      |

(continued)
| Statin          | Dose mg/day | Duration | n   | Pathologies               | Results                                                                 | References |
|----------------|-------------|----------|-----|----------------------------|------------------------------------------------------------------------|------------|
| Simvastatin    | 40          | 8 weeks  | 50  | Metabolic syndrome        | Reduced PAI activity. No alter sP-selectin                            | [148]      |
| Atorvastatin   | 10 and 80   | 30 weeks | 217 | Type 2 diabetes mellitus  | Atorvastatin dose dependently reduced D-dimer and PAI, Fibrinogen, vWF, tPA, FVIIa were not influenced by atorvastatin | [149]      |
| Atorvastatin   | 40          | 10 weeks | 30  | Diabetes                  | Reduction in thrombin generation                                      | [150]      |
| Atorvastatin   | 80          | 2 months |     | Type 1 diabetes, dyslipidemia | Increased fibrin network permeability and decreased thrombin potential, GP IIIa, p-selectin and TF | [151]      |
| Pravastatin    | 40          | 8 weeks  | 50  | Type 2 diabetes           | Reduction in vWF, sTF. Fibrinogen and D-dimer did no decrease          | [152]      |
| Simvastatin    | 20          | 24 weeks |     | Prediabetes and hypercholesterolemia | Decreased PAI levels. Combined therapy with ezetimibe induced reduction in E-selectin, ICAM-1 | [153]      |
| Simvastatin    | 20          | 3 months | 20  | Type 2 diabetes and dyslipidemia | t-Pa increased but also PAI, vWF, E-P-selectin, ICAM-1 and VCAM-1 after simvastatin treatment | [154]      |
| Simvastatin    | 40          | 90 days  | 39  | Hypertriglyceridemia      | Reduced plasma levels and activity of fibrinogen, FVII and PAI-1. No reduction in vWF | [155]      |
| Simvastatin    | 40          | 90 days  | 104 | Hypercholesterolemia      | Simvastatin reduced plasma/activity of fibrinogen, FVII, FX, vWF and PAI. Combined therapy showed the best results in hemostatic variables | [156]      |
| Simvastatin    | 40          | 28 days  | 20  | Hypercholesterolemia      | Decreased TAT, sP-selectin, thrombin in microvascular injury site, B-thromboglobulin | [157]      |
| Atorvastatin   | 80          | 1 month  | 45  | Hypercholesterolemia      | Rosuvastatin normalized platelet cholesterol, TF and FXa production and increased HDL-C | [158]      |
| Rosuvastatin   | 20          |          |     |                            |                                                                        |            |
| Simvastatin, atorvastatin, cerivastin, fluvastatin and pravastatin | N/A | 6 weeks  | 144 | Hypercholesterolemia      | Simvastatin and atorvastatin decreased platelet dependent thrombin generation | [159]      |
regulation of hemostasis considering that D-dimer remains invariable when compared with heparin group [131]. However, in patients with suspected pulmonary embolism, the use of statins, promoted a reduction in D-dimer levels by 15%. Interestingly, the 72% of patients received lipophilic statins (simvastatin, atorvastatin or fluvastatin) presented lower D-dimer levels, but this difference was not significant when compared with hydrophilic statins [132]. Based in the above findings, further studies are required for the exhaustive evaluation of coagulation- and fibrinolysis-related parameters after long term rosvustatin u other statins use.

### 2.3 Statins in Vascular and Cardiovascular-Related Diseases

Statins are also used in cardiovascular-related disorders, where atorvastatin is the most used drug. Interestingly, for depicting the effect of atorvastatin in hemostasis, the administered dose, the exposition time and the concomitant pathology are relevant. In arterial occlusive disease for instance, the administration of atorvastatin at high dose (80 mg/day) reduced plasma prothrombin 1 + 2 fragment concentration, thrombin generation (including thrombin peak) and TF, GPIIIa, P-selectin expression in a mechanism dependent on platelet-derived microparticles, a phenomenon evaluated with complementary in vitro studies [137]. Similarly, an inferior dose of atorvastatin (20 mg/day) in patients with carotid atherosclerotic plaque promoted reduced TF antigen levels and activity [138], suggesting that independent of administered dose, atorvastatin is an effective alternative in the prevention of thrombotic events mediated by TF.

Alternatively, in patients suffering heart failure, a condition known for promoting a procoagulant state, atorvastatin (10 mg/day) for 4 weeks altered endothelial and hepatic proteins involved in coagulation and fibrinolysis, particularly, decreasing antithrombin III, protein-C, FV, tPA and PAI no affecting vWF, FVII and protein-S levels. It is interestingly the authors discussed the anti-inflammatory effect of atorvastatin, founded on the decrement of proinflammatory cytokines, which could be related with alteration in coagulation parameters, and represent other mechanistic pathway in coagulation disorders improvement [134]. Similarly, the
same (10 mg/day) or even a higher dose of atorvastatin (80 mg/day) used on another cohort of patients with acute coronary syndrome for 2 weeks was effective in the modulation of vWF levels [135].

Likewise, in a longer intervention in patients with unstable angina, the administration of atorvastatin at low dose also prevented the increase of antithrombin III and vWF, remain unchanged the levels of tPA, protein-S, FVIII and protein-C. It is important to highlight that the study was conducted in normocholesterolemic patients, which means that the effects on coagulation parameters are independent of cholesterol level improvement, positioning this statin even at low dose as a potential therapeutic alternative in the prevention of thrombotic events [136]. Further, in 19 patients with myocardial infarction treated with 40 mg/day of simvastatin, was observed a reduction in thrombin generation and platelet activation compared with 34 patients unexposed to statins [145]. Finally, in a study comparing the effectiveness of statins in hemostatic regulation of patients with coronary heart disease, results demonstrated that atorvastatin surpasses simvastatin in the improvement of fibrinolytic activity in patients with coronary heart disease, increasing D-dimer levels and tPA activity. Unlike the studies above, this work evaluated a long-time treatment (one year) [146].

Similarly, the use of rosuvastatin in a clinical trial including 53 patients experiencing acute coronary syndrome demonstrated that a high dose (40 mg/day) and short exposition time (8–24 h) reduced platelet-neutrophil and platelet–monocyte interactions without affecting platelet aggregation or p-selectin expression [139]. The use of pravastatin in patients with coronary artery disease and cholesterolemia showed that its administration for 6 months decreased PAI-I but also tPA levels and interestingly, D-dimer, prothrombin, FVII, or vWF production was no affected [141]. Finally, when fluvastatin was administered for 6 h in patients with acute coronary syndrome it promoted an increased expression of endothelial protein-C receptor and TFPI, modifying key antithrombotic factors in truly short periods [142]. Conversely, the use of simvastatin at 80 mg/day for 14 days in a randomized controlled trial of patients with aneurysmal subarachnoid hemorrhage, it was found that this drug shows was not involved in the regulation of coagulation, fibrinolysis, or endothelial function parameters, but reduced, as expected, the levels of cholesterol in blood [140]. This is remarkably interesting considering the opposite results described above, where statins demonstrated key participation in thrombotic events but not in the hemorrhagic one, and considering the pathophysiological differences of these processes, the use of statins in the treatment of hemorrhagic diseases deserve further explorations.

2.4 Metabolic Diseases and Related Risk Factors

In patients with metabolic syndrome, complementary therapy with statins has also showed controversial effects. In a cohort of 88 patients high atorvastatin dose (80 mg/day) for 12 weeks showed to improve lipidic profile without affecting the coagulant activity after 12 weeks of treatment [147]. Conversely, in a cohort of 50 patients, the use of simvastatin (40 mg/day) for 8 weeks showed an improvement in fibrinolysis, through PAI-I activity reduction [148].

In a study performed on diabetic patients, atorvastatin treatment contributed to an improved coagulant status. When the drug was administered at low and high dose (10 and 80 mg/day, respectively) for 30 weeks for instance, it was observed a reduction in D-dimer and PAI-I, and this change was dependent on the administered dose. Also, atorvastatin showed to increase fibrin permeability, decrease thrombin potential, and reduce GPIIIa, p-selectin and TF expression [149]. Similarly, atorvastatin administration at 40 mg/day for 10 weeks reduced thrombin generation [150]. That could be mediated by the reduction in the anticoagulant protein C expression observed in diabetic patients without macrovascular complications [166]. The data above suggest the potential of atorvastatin to improve a procoagulant state through reduction...
in thrombin activity or concentration. Likewise, simvastatin was evaluated in diabetic patients with dyslipidemia and the treatment for 3 months showed to increase fibrinolytic-related proteins as tPA and PAI-I, but also procoagulant factors such as vWF, and cellular adhesion proteins as E-selectin, P-selectin, ICAM-1, and VCAM-1 [154]. On the contrary, the treatment for 24 weeks in pre-diabetic and hypercholesterolemic patients decreases PAI-I levels [153]. This could suggest a time dependent effect and the contribution of baseline conditions as determinant in coagulation outcomes, but further investigation is needed to determine if these variables are relevant.

Moreover, in patients with lipid disorders, simvastatin at 40 mg/day is used, showing a reduction in fibrinogen, FVII and PAI-I levels in plasma after 90 days of treatment in a cohort of 39 patients [155]. Similarly, in a cohort of 104 patients, 90 days of treatment with simvastatin showed to reduce procoagulant proteins as fibrinogen, FVII, FX, vWF and PAI-I [156]. Finally, a study comparing atorvastatin and simvastatin effect, showed differential contribution of these drugs. Both exhibited prolonged PT but only simvastatin reduced fibrinogen concentration and promoted PTT prolongation [160]. Interestingly, simvastatin use showed to increase TAT, sP-selectin, thrombin and β-macroglobulin, essential proteins in platelet function [157], suggesting that platelets present a particular response to statins administration. In this matter, a study comparing the effectiveness of atorvastatin and rosuvastatin in hypercholesterolemic patients showed that rosuvastatin but no atorvastatin normalized platelet cholesterol content and was associated with improved TF and FXa production. The article discusses about a possible pleiotropic effect of rosuvastatin and provide clues about the differential effect of statins [158].

Furthermore, a study comparing simvastatin, atorvastatin, cerivastatin, fluvastatin and pravastatin, showed that only rosuvastatin and atorvastatin decreased platelet dependent thrombin generation [159]. Finally, in hypercholesterolemic patients with essential hypertension and non-alcoholic fatty liver disease, the treatment with statins reduced platelet aggregation by 16%, thrombin time by 12.2% and increased antithrombin III activity by 3%, prothrombin time by 32%, INR by 25% and thrombin time by 23% [165]. These findings are remarkably interesting, considering that in patients with lipid disorders, statins exhibited strong influence in hemostatic parameters and in the regulation of platelet function.

### 2.5 Other Chronic Diseases

In other chronic diseases as chronic obstructive pulmonary disease (COPD), simvastatin at 40 mg/day promoted a slight increase in TF activity and decreased TFPI and FVII [161]. Moreover, the administration of simvastatin improved clot properties in COPD patients, reduced density of networks and less resistant to lysis [162]. Conversely, in cancer patients, statin (rosuvastatin) use does not improve biomarkers of VTE risk and in patients with chronic kidney disease, statin (atorvastatin) use does not change the fibrinolytic parameters evaluated [164].

### 2.6 Molecular Mechanisms in Statins Improved Coagulation

The evidence summarized here showed that the action of statins in coagulation regulation is highly dependent on statin subtype used, administered dose, and target tissue [79, 167], although involved molecular mechanisms are poorly understood (Fig. 2.2).

The liver, the main site of cholesterol synthesis and subsequently the main target of statins [96, 168] is also the primary site of coagulation factors synthesis [169]. Therefore, deciphering local mechanisms of coagulation regulation mediated by statins is a relevant issue. Recent findings support this notion considering that hepatic cell cultures and animal models showed that Rac1/hepatocyte nuclear factor 1α (HNF1α) pathway participated in the increased expression
of the anticoagulant protein C after simvastatin treatment [67]. Despite this interesting finding, more studies are needed to elucidate the interplay between statins, and hepatic-derived coagulation regulation.

Given the participation of platelets in hemostasis [170], it is necessary deepen in the molecular mechanisms explaining the effect exerted by statins since previous findings indicated that statins reduce mean platelet volume [171] and reduce platelet reactivity and thus, improving in 65% aspirin resistance [172]. Interestingly, experiments in pre- or post-treated platelets with lovastatin showed reduction in platelet hyperreactivity, prevention in thrombin generation, downregulation in TF expression and reduced platelet aggregation [158]. Complementary studies explored the effects of pravastatin, atorvastatin and simvastatin on platelet reactivity and found that only pravastatin and simvastatin reduced platelet aggregation and thromboxane formation by platelet membrane-associated proteins [173]. The protease-activated receptor-1 (PAR-1), with a pivotal participation in platelet activation, has demonstrated high responsiveness to statins, particularly pravastatin that dose-dependently downregulated platelet activity by preventing ADP-induced PAR-1 expression [174]. Finally, statins including atorvastatin, rosuvastatin and fluvastatin potentiate the antibacterial effect of platelets over Staphylococcus aureus through glycoprotein IIb-IIIa, however, the implication of this finding requires further in vivo exploration [175].

It is well known that hypercholesterolemia promoted platelet reactivity associated with a procoagulant state and increased CVD risk [176]. Interestingly, high ox-LDL levels in hypercholesterolemic mice lead upregulation in monocyte-derived TF expression and activity, increasing thrombin-antithrombin activity and D-dimer levels, both markers of systemic coagulation activation. Interestingly, hypercholesterolemic mice and monkeys treated with simvastatin 50 mg/kg/day and 10 mg/kg/day respectively, reduced ox-LDL levels and monocyte-derived TF, with subsequent attenuation of plasma coagulation activation markers independent of total cholesterol levels regulation [177].

Endothelial dysfunction and concomitant microthrombosis are key mechanisms in the
pathogenesis of sepsis [178]. During experimental sepsis was demonstrated that simvastatin (25 mg/kg/day) pretreatment prevented hepatic microthrombosis and peripheral consumption-related bleeding by preserving thrombomodulin expression and downregulate fibrin deposition and vWF release in liver sinusoids [179]. Similarly, simvastatin (20 mg/kg/day) administration reduced septic-induced coagulopathy in mice, improving coagulation factors and platelet depletion, PAI-1 expression and fibrin deposition, ultimately, attenuating disseminated intravascular coagulation and increasing survival rate. Although in this case, simvastatin effects was also attributed to improved intestinal permeability and dysbiosis [180].

Endothelial-derived molecules as nitric oxide has been directly associated with reduced platelet adhesion to endothelial cells [181] and inactivation of intravascular coagulation [182]. In that context it is interesting the contribution of statins, considering that simvastatin promotes e-NOS activation [183] through Akt activation [184] and, active metabolites of atorvastatin have shown reduced oxidative stress in endothelial cells, increasing NO bioavailability [185]. The above has direct implications in endothelial function, considering NO decrease endothelial adhesion proteins, including ICAM-1 [186]. Complementary studies in endothelial cells pre-incubated with simvastatin or atorvastatin, stimulated with LPS y cocultured with platelets find reduced expression of VCAM-1 [187].

Considering the inconclusive and controversial evidence of statins contribution in the improvement of fibrinolysis, the effectiveness of atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin and simvastatin in vascular cells was evaluated. Results showed that all statins except pravastatin promoted a significant decrease in PAI-1 mRNA and protein in smooth muscle cells (SMCs) and ECs under IL-1 or TNF-alpha stimulation. Similarly, all statin, except pravastatin upregulated tPA release in SMC, whereas in endothelium, only simvastatin and lovastatin elevated tPA mRNA and protein production [188]. In the endothelial cell line EA.hy926 was demonstrated that statins, specifically atorvastatin promoted antithrombotic properties through the regulation of cholesterol and lipid metabolism, since atorvastatin administration at micromolar concentrations reduced PS, sphingomyelin, cholesterol and ceramides between 50 and 70%, that associated to significant reduction in FVIIa, TF and prothrombinase activity, suggesting that statins limit enzymatic proteolytic complexes involved in exacerbated coagulation cascade propagation [189].

Finally, endothelial progenitor cells (EPCs) with an essential role in the regulation of platelet activation, aggregation, and subsequent participation in thrombus formation [190, 191], showed a significant increase after atorvastatin administration, showing a novel and promising role in hemostasis regulation [192].

### 2.7 Concluding Remarks

Taken together, evidence from clinical research showed that statins are proved and effective regulators of dyslipidemia with a high impact in vascular function and hemostasis regulation in acute and chronic diseases. However, relevant primary investigation is required to elucidate the underlying molecular mechanisms mediating differential statin actions, improving their therapeutic actions and avoid detrimental side effects.

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Effects of Adrenergic Receptor Stimulation on Human Hemostasis: A Systematic Review

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Abstract

Catecholamine stimulation over adrenergic receptors results in a state of hypercoagulability. Chronic stress involves the release and increase in circulation of catecholamines and other stress related hormones. Numerous observational studies in human have related stressful scenarios to several coagulation variables, but controlled stimulation with agonists or antagonists to adrenergic receptors are scarce. This systematic review is aimed at presenting an updated appraisal of the effect of adrenergic receptor modulation on variables related to human hemostasis by systematically reviewing the effect of adrenergic receptor-targeting drugs on scale variables related to hemostasis. By searching 3 databases for articles published between January 1st 2011 and February 16th, 2022 reporting effects on coagulation parameters from stimulation with $\alpha$- or $\beta$-adrenergic receptor targeting drugs in humans regardless of baseline condition, excluding records different from original research and those not addressing the main aim of this systematic review. Risk of bias assessed using the Revised Cochrane risk-of-bias tool for randomized trials (RoB 2). Tables describing a prothrombotic anti-fibrinolytic state induced after $\beta$-adrenergic receptor agonist stimulation and
the opposite after α1-, β-adrenergic receptor antagonist stimulation were synthesized from 4 eligible records by comparing hemostasis-related variables to their baseline. Notwithstanding this low number of records, experimental interventions included were sound and mostly unbiased, results were coherent, and outcomes were biologically plausible. In summary, this systematic review provides a critical systematic assessment and an updated elaboration, and its shortcomings highlight the need for further investigation in the field of hematology.

**Keywords**

Adrenergic • Coagulation • Hemostasis • Catecholamines • Fibrinolysis

**Abbreviations**

- aPTT: Activated partial thromboplastin time
- BPM: Beats per minute
- cAMP: Cyclic adenosine monophosphate
- CREB: CRE binding protein
- DAG: 1,2-diacylglycerol
- GDP: Guanosine diphosphate
- GPCR: G protein-coupled receptors
- GTP: Guanosine triphosphate
- HCTZ: Hydrochlorothiazide
- HUVEC: Human umbilical vein endothelial cells
- IP3: Inositol 1,4,5-trisphosphate
- LVH: Left ventricle hypertrophia
- MPV: Mean platelet volume
- PCGR: Protein C global ratio
- PKA: cAMP-dependent kinase
- PKC: Calcium-dependent protein kinase
- PKC: Protein kinase C
- PLC: Phospholipase C
- PRISMA: Preferred reporting items for systematic reviews and meta-analyses framework
- PTF1 + 2: Prothrombin fragments 1 + 2
- RoB 2: Revised Cochrane risk-of-bias tool for randomized trials
- tPA: Tissue plasminogen activator
- vWF: von Willebrand Factor
- WoS: Clarivate Analytics’ Web of Science
- WPBs: Weibel–Palade bodies

### 3.1 Introduction

Adrenaline and noradrenaline, the hormone and the neurotransmitter respectively, are produced from L-tyrosine in two sources in the body: sympathetic nerve endings, which release noradrenaline on effector organs after stimulation, and the chromaffin cells of the adrenal medulla, which are actually sympathetic postganglionic neurons and the main source of synthesis, storage and release of adrenaline and noradrenaline under the stimulation of nicotine acetylcholine receptors [1].

Both catecholamines (adrenaline and noradrenaline), named as such because of the catechol ring they share in their chemical structure (1,2-dihydroxybenzene), mediate a wide variety of essential functions in the body, among which vasodilation, vasoconstriction, cardiac proliferation, blood pressure, and systolic and diastolic pressure are the most prominent [2, 3]. These catecholamines are released in situations of anger, stress, or anxiety, and during allergic or hypotensive reactions, mediated by other hormones such as histamine, angiotensin II, and bradykinin [1]. They exert their effects by binding to adrenergic receptors or adrenoreceptors, which are distributed in the central and autonomic nervous systems, as well as in peripheral tissues [2, 3]. The importance of these chemical mediators is such that the alteration in the synthesis and release of catecholamines—which circulate normally in the blood in the order of nanomoles [1]—or in the way they bind to their specific receptors can lead to cardiac and prostatic hyperplasia, cardiac hypertrophy, hypertension or even cancer [2].
3.1.1 Classification of Adrenergic Receptors

Based on their pharmacological affinity to a myriad of drugs and synthetic agents (agonists and antagonists), adrenergic receptors are classified into three groups: $\beta$, $\alpha_1$ and $\alpha_2$, a nomenclature owed to the identity in their primary sequence [2, 3]. Each group has three subtypes: $\beta_1$, $\beta_2$, $\alpha_1A$, $\alpha_1B$, $\alpha_1D$, $\alpha_2A$, $\alpha_2B$ and $\alpha_2C$, all products of different genes. The $\alpha$-adrenergic receptors are generally considered vasoconstrictors, while $\beta$-adrenergic receptors are mainly considered vasodilators. The fewest $\alpha$-receptors are found in the cerebral vessels. The $\beta_1$ and $\beta_2$ receptors are mostly found in the vessels of the heart, lungs, and mesentery. In the myocardium, the inotropic and chronotropic effects of the catecholamines, are mediated most strongly via $\beta_1$ receptors.

Sutherland and Rall observed that cyclic adenosine monophosphate (cAMP), which forms from the cyclization of ATP, is an intermediary in the action of adrenaline, which acts on the phosphorylase that participates in the formation of glucose from glycogen [4]. In 1967, two types of $\beta$-adrenergic receptors were identified after observations on their difference in affinity for adrenaline, which activates the $\beta_2$-adrenergic subtype with 100 times more potency than the $\beta_1$-adrenergic subtype. The $\beta_3$-adrenergic subtype was discovered later and was considered “atypical” because of its relative insensitivity to typical $\beta_1$ and $\beta_2$ antagonists [5].

The classification of $\alpha_1$-adrenergic receptor began with the use of prazosin, a selective antagonist [2]. With WB4101, another antagonist, two subtypes of $\alpha_1$-adrenergic receptor were clearly identified: $\alpha_1A$ and $\alpha_1B$. The first gene cloned was that of the $\alpha_1B$ receptor, later the $\alpha_1A$-adrenergic receptor was cloned, with which there was an initial controversy since it had previously been named $\alpha_1C$-adrenergic ($\alpha_1A/C$). Subsequently, the $\alpha_1D$-adrenergic receptor ($\alpha_1A/D$) was identified and cloned, consolidating the nomenclature as $\alpha_1A$, $\alpha_1B$ and $\alpha_1D$. On the other hand, the group of $\alpha_2$-adrenergic receptors was finally identified using the antagonist yohimbine, and classified into two subtypes: $\alpha_2B$ and $\alpha_2C$, characterized as agonists and antagonists in functional and biochemical assays [2, 3].

3.1.2 Characteristics of Adrenergic Receptors

Adrenergic receptors belong to the superfamily of G protein-coupled receptors (GPCR). They are comprised of seven hydrophobic regions or transmembrane helices, three extracellular loops essential for ligand binding, and three intracellular loops. Adrenergic receptors respond to changes in the environment and different physiological conditions, such as an changes in the concentration of adrenaline and noradrenaline in the bloodstream [1]. The binding of an agonist to the receptor induces a conformational change that allows it to couple to the heterotrimeric G protein, which, when activated, exchanges guanosine diphosphate (GDP), bound to the $\alpha$-subunit in its inactive state, for guanosine triphosphate (GTP). Release of GDP allows dissociation of the GTP-bearing $\alpha$-subunit from the $\beta\gamma$ dimer. Hydrolysis of GTP to GDP by the $\alpha$ subunit (which has GTPase activity) allows the $\alpha$ and $\beta\gamma$ subunits to associate; the activation/deactivation cycle of the G protein starts again when the receptor is activated [5].

There are four families of heterotrimeric G proteins: $G_s$ (stimulatory), $G_i$ (inhibitory), $G_{q/11}$ and $G_{12/13}$ [5, 6]. After the activation of the G protein ($G_{q/11}$ and $G_i$), phospholipase C (PLC) catalyzes the release of inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), which cause the release of calcium ions from the endoplasmic reticulum; while adenyl cyclase produces cyclic AMP (cAMP). These second messengers (IP3, DAG, calcium ions, and cAMP) activate proteins that amplify the extracellular signal, such as calcium-dependent protein kinase (PKC) and cAMP-dependent kinase (PKA) [5].
3.1.3 Tissue Distribution of Adrenergic Receptors

Adrenergic receptor expression in human tissues has been reported to have significant differences with other animals [7, 8]. Classical studies describe the most predominant subtype of $\alpha_1$-adrenergic receptor in human tissues to be the $\alpha_{1C}$-adrenergic receptor [9], which predominates in the liver, heart, vena cava, cerebellum, and cerebral cortex; followed by the $\alpha_{1B}$-adrenergic receptor, which predominates in the kidney and spleen; then followed by the $\alpha_{1D}$-adrenergic receptor, which predominates in the aorta. Beta-adrenergic receptor subtypes $\beta_1$, $\beta_2$, and $\beta_3$, have also been identified predominantly in cardiac, airway smooth muscle, and adipose tissue, respectively [10]. However, a recent and comprehensive quantitative analysis of the human transcriptome reported $\alpha_{1B}$-adrenergic receptor expression to be predominant in the spleen, brain, and kidney, $\alpha_{2A}$-adrenergic receptor expression to be predominant in the adipose tissue, gallbladder, and pancreas, $\alpha_{2B}$-adrenergic receptor expression to be predominant in the adipose tissue, esophagus, and lung, $\beta_2$-adrenergic receptor expression to be predominant in the adipose tissue, gallbladder, and pancreas, $\beta_3$-adrenergic receptor expression to be predominant in the spleen, adipose tissue, and liver [11]. Because the focus of this latter analysis is not on adrenergic biology, the list of mRNA and proteins, although extensive and thorough, is not sufficient to cover the entirety of the adrenergic receptor family. Thus, in light of the discrepancies found in the latter quantitative analysis, a technological gap appears, which encourages further research on adrenergic receptor distribution. This differential tissue distribution highlights the level of complexity of adrenergic receptor expression and suggests that some selectivity may be obtained using adrenergic receptor subtype-selective agonists and antagonists for treating various human diseases.

3.1.4 Physiological Activity of Adrenergic Receptors

Catecholamines influence all major organs of the body. Its effects take place in seconds compared to the minutes, hours or days that characterize the actions of the endocrine system. Catecholamines participate in integrative mechanisms, both neural and endocrine. Its participation in the response to stress, the regulation of smooth muscle tone, the control of blood pressure, the metabolism of carbohydrates and lipids, thermoregulation, as well as in the secretion of various hormones and in numerous central and peripheral processes [5]. The adrenergic system also plays a fundamental role with the hypothalamic–pituitary–adrenal axis (CRH-ACTH-Cortisol), in responses to stress.

The sympathetic activity of the nervous system and the secretion of catecholamines from the adrenal medulla are often coordinated, although their actions are not always simultaneous and congruent. In many situations of intense sympathetic stimulation, such as exercise or exposure to cold, the adrenal medulla is progressively stimulated and circulating adrenaline reinforces the physiological effects of the sympathetic nervous system. However, in other situations they act independently, e.g., when hypoglycemia stimulates the adrenal medulla as a defense mechanism, to compensate the hypoglycemic state, or when hypotension stimulates the sympathetic nervous system to maintain blood pressure and allow for tissue perfusion [5].

3.1.5 Therapeutic Modulation of Adrenergic Receptor-Signaling

Catecholamines, and their signaling through adrenergic receptors in target cells, base their effect most prominently to control hemodynamics. However, there are effects that extend
beyond the cardiovascular (e.g., in the gastroin-testinal tract, lungs, immune system, endocrine system, bone marrow, muscles, metabolism, and the coagulation system) that become of particular interest during chronic, excessive, or therapeutic sympathetic stimulation [12, 13].

Therapeutically, catecholamines are mainly used in case dominated by a reduction in hemodynamic capacity, most notably during shock, where the hemodynamic deficit hinders the capacity of the body to adequately cover the blood supply to organs. In practice, shock is often defined as a drop in systolic or mean arterial blood pressure below certain limits (e.g., 90 or 65 mmHg). Since arterial blood pressure is feasible to monitor, such limit values have often been established as a trigger for therapy with vasopressors. For the treatment of hypotension during septic shock, the current guidelines [14] recommend the use of norepinephrine in addition to sufficient volume therapy, while adrenaline is primarily recommended in anaphylactic shock [15]. The administration of vasopressors increases systemic vascular resistance and thus the afterload of the heart. Additionally, constriction of the venous capacity vessels in the event of a volume deficiency can also increase the preload and thus the stroke volume via the Frank–Starling mechanism. In the case of cardiogenic shock, the clinically established definition includes not only hypotension but also other hemodynamic measurements [16]. In such situations, clinical guidelines recommend the administration of norepinephrine to increase mean arterial pressure to a range between 65 and 75 mmHg.

Such therapeutic algorithms have significantly shaped the procedures in intensive care units. However, there has been increasing evidence that the administration of catecholamines can also adversely affect patient prognosis or that catecholamines can have a damaging effect on target organs, e.g., the heart and microcirculation. In this context, the hemodynamic target parameters, on which catecholamine therapy is mainly based, have since been questioned [17–19].

### 3.1.6 Chronic Stimulation of Adrenergic Receptors

Paradoxically, evidence on the effects of chronic, persistent catecholamine stimulation outside of the intensive care unit is remarkably scarce. Interestingly, it has been described that long-term sympathetic activation leads to β-adrenergic receptor desensitization and decreased expression (downregulation) [20]. However, few studies have regarded chronic catecholamine stimulation at a body-wide level, of which a causal link has been established with Alzheimer’s disease, dementia and amnestic mild cognitive impairment [21], posttraumatic stress disorder [22], cardiac hypertrophy [23], cancer [24, 25], immunosuppression [26, 27], and overall inflammatory and procoagulant state [28]. Interestingly, the greater fraction of current knowledge about adrenergic physiology focuses on the acute, immediate, effects of adrenergic receptor modulators [5] rather than on their chronic effects, and amongst them, hemostasis is the least studied. Thus, the objective of this systematic review is to present an updated appraisal of the effect of adrenergic receptor modulation on variables related to human hemostasis by systematically reviewing the effect of adrenergic receptor-targeting drugs on scale variables related to hemostasis.

### 3.2 Methods

This systematic review was reported using the preferred reporting items for systematic reviews and meta-analyses framework (PRISMA) [29].

**Eligibility criteria:** Overall inclusion criterion was: articles reporting effects on coagulation parameters from stimulation with α- or β-adrenergic receptor targeting drugs in humans regardless of baseline condition. Exclusion criteria were: articles different from original research, not addressing the objective, not reporting
baseline and after-treatment measurements, and not reporting statistically significant differences (Fig. 3.1). Remaining records included for qualitative analysis were classified into two groups according to the effect of the drug administered (agonists and antagonists).

**Fig. 3.1** PRISMA 2020 flow diagram for study selection
Information sources were 3 databases queried from January 1st, 2011 until February 16th, 2022: National Library of Medicine’s PubMed, Elsevier’s Scopus and Clarivate Analytics’ Web of Science (WoS). Search strategy for each database is fully detailed in Table S1. In brief, a combination of keywords (i.e., adrenergic receptor, D-dimer, fibrinogen, and coagulation) was used in search strings limiting records to those in English within the date frame. For the selection process, two reviewers (DA, SG) worked independently on each record without automation assistance during the selection process, which was based on carefully reading the title, abstract, and/or full text, to exclude those different from original research that eluded the search filter, to exclude those not addressing the objective, and those reporting data without statistically significant differences (Fig. 3.1). Discrepancies were then sorted out over a discussion round including all authors and led by both reviewers. Data was then collected independently by the reviewers (DA, SG) from texts, tables, or figures, of eligible records.

Eligible records were those that presented the net effect of adrenergic receptor-targeting drugs on scale variables related to hemostasis (i.e., coagulation or fibrinolysis) within the results. Outcomes sought were either a significant increase or decrease of the scale variable with respect to its baseline. This operational arrangement ensured full compatibility between all the results and the outcomes. Complementary data on baseline condition, dose, and readout time, were also considered for evaluation but were not considered as part of the outcomes. Given its complete availability both, outcomes and complementary data, were neither imputed nor assumed.

Risk of bias and certainty assessment: For each record included, risk of bias was assessed independently by two reviewers (DA, SG) using the Revised Cochrane risk-of-bias tool for randomized trials (RoB 2) [30] without using any automation. Also, for every record included, certainty was assessed independently by two reviewers (DA, SG) using the CASP Randomised Controlled Trials Checklist [31] without using any automation. Appraisal for missing results was addressed using both tools. Results, comments, and discrepancies from these assessment tools were placed and resolved in a discussion round including all authors and led by both reviewers (DA, SG).

Synthesis: For the synthesis of the results, plausibility tests to obtain effect measures (i.e., risk ratio and mean difference) for each scale variable were performed first. Then, scale variables were compared to their baseline, outcomes were constructed, and complementary data (i.e., baseline condition, target adrenergic receptors, dose, and readout time) was matched. Lastly, data was arranged on the basis of the mechanism of stimulation of adrenergic receptor-targeting drugs: agonists (Table 3.1) and antagonists (Table 3.2).

Table 3.1 Effect of AR agonists on variables related to human hemostasis

| Baseline condition | Drug    | Mechanism | AR | Dose   | Readout time | Outcome | Hemostasis-related scale variable | Reference |
|-------------------|---------|------------|----|--------|--------------|---------|----------------------------------|-----------|
| Healthy           | Salbutamol | Agonist    | $\beta_1$, $\beta_2$ | 5 mg Single dose | 60 min | Increased | Factor V activity Factor VIII activity vWF antigen D-dimer PTF1 + 2 | [32]      |
|                   |         |            |    |        |              |         | Decreased | aPTT PCGR tPA |          |

Abbreviations AR adrenergic receptor; vWF von Willebrand factor; PTF1 + 2 prothrombin fragments 1 + 2; aPTT activated partial thromboplastin time; PCGR Protein C global ratio; tPA tissue plasminogen activator
3.3 Results

The search identified 1025 total records, of which, 1020 were excluded during the selection process and 4 were included (Fig. 3.1). Because the search was performed over three different sources (PubMed, Scopus, and WoS) records duplicated across databases (188) were excluded. Although filters for article type (original research) were applied, post hoc manual screening of meta-information further identified 148 records not meeting this inclusion criterion. Full-text analysis identified 675 records not addressing the objective and 555 records not providing data on baseline levels of scale variables related to hemostasis for comparison, and out of those that did, 8 records reported differences that were not statistically significant. Because of the way inclusion criteria were designed, the search resulted to be deterministic, i.e., without any records borderline for exclusion.

3.3.1 Study Characteristics

The 4 records identified in the search were prospective randomized interventional studies with a single recruitment step except for article by Boman et al. [33] which was an interventional sub-study performed in a sample already recruited for a larger trial, which, in turn, had already been randomized. Baseline subject characteristics were mild hypertension and left ventricular hypertrophy [33], prostatic hypertrophy [34], coronary angiopathy [35], and one study of generally healthy subjects [32].

Average subject age was 60 years old, except for article by Ali-Saleh et al. [32], for which

| Table 3.2 | Effect of AR antagonists on variables related to human hemostasis |
|-----------|------------------------------------------------------------------|
| **Baseline condition** | **Drug** | **Mechanism** | **AR** | **Dose** | **Readout time** | **Outcome** | **Hemostasis-related scale variable** | **References** |
| M-m hypertension and LVH | Atenolol | Blocker | $\beta_1$ | 50 mg per day | 36 weeks | Increased | tPA | vWF |
| Benign prostatic hyperplasia | Doxazosin | Blocker | $\alpha_1$ | 4 mg per day | 12 weeks | Increased | Bleeding time | [34] |
| | Terazosin | | | 5 mg per day | | | Coagulation time | |
| | Doxazosin | | | 4 mg per day | | | | |
| | Terazosin | | | 5 mg per day | | | | |
| Chronic angiopathy | Carvedilol | Blocker | $\alpha_1, \beta$ | 25 mg per day | 6 months | Decreased | D-dimer | [35] |
| | Nebivolol | | $\beta_1$ | 10 mg per day | | | MPV | |
| | Carvedilol | | $\alpha_1, \beta_1, \beta_2$ | 25 mg per day | | | | |
| | Nebivolol | | $\beta_1$ | 10 mg per day | | | | |

*Abbreviations AR adrenergic receptor; M-m mild to moderate; LVH left ventricle hypertrophia; tPA tissue plasminogen activator; vWF von Willebrand factor; MPV mean platelet volume*
average subject age was 26 years old. Average systolic blood pressure was 155.5 mmHg, average diastolic blood pressure was 95.5 mmHg, and average heart rate was 76.75 BPM across studies, except for the only study including healthy subjects [32] (for which average systolic blood pressure was 116 mmHg, average diastolic blood pressure was 69 mmHg, and average heart rate was 68 BPM) and article by Alan et al. [34], for which baseline biopotentials were recorded but failed to be reported.

A summary of the adrenergic receptor-targeting drugs administered, their mechanism, dose, and readout times, is available in Tables 3.1 and 3.2. Blood sample handling was standard across all studies and hemostasis-related scale variables were measured using clinical-grade equipment and commercially available ELISA kits.

### 3.3.2 Risk of Bias

Critical appraisal of the findings in the 4 records identified in the search revealed a low risk of bias on four domains: (A) Sequence for participant allocation was explicitly random in most of the records. Baseline differences between intervention groups did not suggest a problem with the randomization process. (B) Data was available for all, or nearly all, participants, (C) Methods of measuring continuous variables were appropriate, and (D) Numerical results were unlikely to have been selected on the basis of results within the outcomes. A low risk of bias was also found on a fifth domain (E, Appraisal of deviations from the intended interventions) for all records except one [33]. Study by Boman et al. [33], which included subjects with mild hypertension and left ventricular hypertrophy at baseline, set a goal blood pressure (< 140/90 mmHg) to reach during a follow-up period of 12 weeks. During this period, an unspecified number of the patients (those not reaching goal blood pressure after 6 or 12 weeks of treatment with the original intervention drug) had a secondary intervention with hydrochlorothiazide (HCTZ) to help them reach such goal. Risk of bias due to missing results was low, as none of the studies failed to report results.

### 3.3.3 Results of Individual Studies and Synthesis

With on a sample of 51 participants who had benign prostatic hyperplasia, Alan et al. [34] administered $\alpha_1$-adrenergic receptor blockers for 12 weeks. Participants who were administered doxazosin ($n = 25$) showed a significant ($p < 0.05$) 22.9% increase in bleeding time from $1.502 \pm 0.39$ to $1.846 \pm 1.93$ min and a significant ($p < 0.05$) 22.13% increase in coagulation time from $4.429 \pm 1.16$ to $5.409 \pm 1.42$ min, measured at baseline and after 12 weeks. Participants who were administered terazosin ($n = 26$) showed a significant ($p < 0.05$) 20.16% increase in bleeding time from $1.384 \pm 0.31$ to $1.663 \pm 0.49$ min and a significant ($p < 0.05$) 10.2% increase in coagulation time from $5.158 \pm 1.13$ to $5.684 \pm 1.18$ min, as measured at baseline and after 12 weeks (Table 3.2).

On a sample of 17 healthy participants, Ali-Saleh et al. [32] administered a $\beta_2$-adrenergic receptor agonist for 60 min. Participants who were administered salbutamol showed a significant ($p < 0.05$) 4.59% increase in Factor $V$ activity from $92.182 \pm 4.22$ to $96.41 \pm 4.25$%, a significant ($p = 0.05$) 12.62% increase in Factor VIII activity from a mean $102.53 \pm 5.85$ to $115.47 \pm 5.62$%, a significant ($p < 0.01$) 171.71% increase in von Willebrand Factor (vWF) antigen levels from a mean $98.06 \pm 6.83$ to $103.24 \pm 6.64$, a significant ($p < 0.05$) 6.81% decrease in activated partial thromboplastin time (aPTT) from a mean $27.37 \pm 0.45$ to $25.51 \pm 0.56$ s, a significant ($p < 0.01$) 171.71% increase in D-dimer from a mean $0.27 \pm 0.04$ to $0.73 \pm 0.24$ mg/L, a significant ($p < 0.05$) 285.43% increase in prothrombin fragments 1 + 2 (PTF1 + 2) from a mean $152.65 \pm 5.85$ to $320.09 \pm 588.35$ pM, a significant ($p < 0.001$) 17.66% decrease in Protein C global ratio (PCGR) from a mean $0.99 \pm 0.07$ to $0.82 \pm 0.06$, and a significant ($p < 0.1$) 7.69% decrease in tissue plasminogen activator (tPA) from a mean $6.5 \pm 0.5$ to $6 \pm 0.4$ ng/mL, as measured at baseline and after 60 min (Table 3.1).

On a sample of 11 participants who had mild-to-moderate hypertension and left ventricle...
hyperplasia, Boman et al. [33] administered a \( \beta_1 \)-adrenergic receptor blocker for 36 weeks. After adjusting for both baseline systolic blood pressure and change in left ventricular mass, participants who were administered atenolol showed a significant (\( p < 0.1 \)) 33.71% increase in levels of tPA from a median 8.9 to 11.9 ng/mL, a significant (\( p < 0.1 \)) 18.41% increase in vWF from 113.5 to 134.3%, as measured at baseline and after 36 weeks (Table 3.2).

On a sample of 61 participants who had chronic angiopathy, Karabacak et al. [35] administered an \( \alpha_1 \)-, \( \beta \)-adrenergic receptor blocker, or a \( \beta_1 \)-adrenergic receptor blocker for 6 months. Participants who were administered carvedilol (n = 31) showed a significant (\( p < 0.01 \)) 13.48% decrease in mean platelet volume (MPV) from a mean 8.9 \( \pm \) 1 to 7.7 \( \pm \) 1 fL, and a significant (\( p < 0.01 \)) 49.07% decrease in D-dimer from a mean 428 \( \pm \) 306 to 218 \( \pm \) 164 \( \mu \)g/dL, as measured at baseline and after 6 months (Table 3.2). Participants who were administered nebivolol (n = 30) showed a significant (\( p < 0.01 \)) 9.09% decrease in MPV from a mean 8.8 \( \pm \) 1.2 to 8 \( \pm \) 0.7 fL, and a significant (\( p < 0.01 \)) 53.74% decrease in D-dimer from a mean 454 \( \pm \) 407 to 210 \( \pm \) 170 \( \mu \)g/dL, as measured at baseline and after 6 months (Table 3.2).

For the synthesis of the results, scale variables were tested for effect measures. However, paucity of comparable variables and pooled sample sizes were insufficient to run statistical tests. Therefore, scale variables were compared to their baseline and outcomes sought were either a significant increase or decrease of such variable. Levels of D-dimer increased with a \( \beta_2 \)-adrenergic receptor agonist and decreased consistently with \( \alpha_1 \)-, \( \beta \)-adrenergic receptor blockers. Levels of IPA decreased with a \( \beta_2 \)-adrenergic receptor agonist and increased with a \( \beta \)-adrenergic receptor blocker (the latter with high risk of bias). Levels of vWF increased with both a \( \beta_2 \)-adrenergic receptor agonist and a \( \beta \)-adrenergic receptor blocker (the latter with high risk of bias). MPV decreased consistently with \( \alpha_1 \)-, \( \beta_1 \)-, and \( \beta_2 \)-blockers. Activity of Factors V and VIII increased and aPTT and PCGR decreased with a \( \beta_2 \)-adrenergic receptor agonist. Bleeding and coagulation times increased with an \( \alpha_1 \)-adrenergic receptor blocker.

### 3.3.4 Assessments of Certainty

Certainty in the body of evidence was critically appraised on the basis of three domains. (A) All 4 records addressed a clearly focused research question, most of them assigned participants randomly, and all of them accounted for the same number of participants at its conclusion. (B) Methodologically, all 4 records included at least one level of blindness to the intervention (except for one [34], for which there is no explicit mention to this point), had similar baseline conditions between groups, and each study group received the same type of intervention (except for one [33], which included an additional level of treatment for select participants). (C) Results reported in all 4 records reported the effects of the intervention comprehensively and with precision.

### 3.4 Discussion

The present systematic review reports an updated appraisal of the effect of adrenergic receptor modulation on variables related to human hemostasis. Overall, a pro-thrombotic anti-fibrinolytic state was induced after \( \beta \)-adrenergic receptor agonist stimulation and the opposite was induced after \( \alpha_1 \)-, \( \beta \)-adrenergic receptor antagonist stimulation. Salbutamol, which is a partial \( \beta_2 \)-adrenergic receptor agonist [36], is able to extend its action on \( \beta_2 \)-adrenergic receptors beyond pulmonary tissues [37]. Thus, the increase in pro-coagulant proteins (Factors V and VIII activity, vWF, D-dimer, and PTF1 + 2) and the decrease of anti-fibrinolytic parameters (aPTT, PCGR, and tPA) is a consistent way to interpret its extrapulmonary effects under a healthy baseline (Table 3.1). The same shows to be true by counterpart with \( \beta_1 \)-\( \beta_2 \)-adrenergic receptor blockers administered \textit{p.o.}, which decrease pro-coagulant parameters D-dimer and...
MPV, increase anti-fibrinolytic parameter tPA, and increase overall coagulation parameters bleeding and coagulation time (Table 3.2). Interestingly, this effect appears to be consistent regardless of the vast differences in readout times (60 min through 6 months).

Agonists to β-adrenergic receptors initiate an intracellular signaling cascade typically involving activation of proteins Gs, adenylate cyclase, and cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA)—the latter via an increase in cAMP, resulting in the release of pro-coagulation proteins. This is best exemplified in endothelial cells, which line blood vessels internally all throughout the body. After β-adrenergic receptor stimulation, the rise in cAMP induces a slow but sustained release of Weibel–Palade bodies (WPBs) and their pro-coagulant contents [38] (significantly vWF, P-selectin, and coagulation Factor VIII) [39], thus explaining the rise in vWF, coagulation factor VIII, and downstream surrogates such as D-dimer and PTF1 + 2, observed in Table 3.1, and the inverse effect observed in Table 3.2. Interestingly, WPBs also respond to rises in intracellular free Ca2+ which occur downstream of a1-adrenergic receptor stimulation through protein Gq, thus explaining why blocking this signaling pathway results in a decrease of coagulation surrogates D-dimer and MPV, and overall coagulation parameters bleeding and coagulation times (Table 3.2). Additionally, increased levels of Factor VIII can be further corroborated by contrast with observations by an independent group who established that Factor VIII plasma concentration can be decreased with the use of β-adrenergic receptor antagonist propranolol [40].

An apparently contradictory situation has been detected for tPA in Tables 3.1 and 3.2. While levels of tPA were expected to increase after β-adrenergic receptor stimulation with an agonist and to decrease with an antagonist because (1) tPA is a component of WPBs, and should share the same outcome as Factor VIII and vWF by means of the β-adrenergic receptor/adenylate cyclase/cAMP signaling pathway [38], and (2) as β-adrenergic receptor stimulation increases intracellular cAMP, activation of highly conserved, species-specific, cAMP response elements (CRE) within the tPA gene promoter via CRE binding (CREB) proteins stimulate tPA synthesis de novo [41], the outcome for β-adrenergic receptor agonism was to decrease and for β-adrenergic receptor antagonism was to increase. Our explanation to this conundrum is that tPA is being actively consumed in the overall pro-coagulant state. Although, naturally, this warrants further experimental considerations, especially because tPA levels have been described as refractory to high levels of cAMP induced by adenylate cyclase agonist forskolin in human umbilical vein endothelial cells (HUVECs) [42], and particularly because levels of tPA have been shown to increase after stimulation of protein kinase C (PKC) with phorbolester [42]. Notably, PKC is an effector of the α1-adrenergic receptor intracellular cascade, and while decreased plasmatic levels of tPA could be associated with active consumption, an overall crosstalk of both signaling pathways cannot be ruled out. In fact, convergence—and multi-level crosstalk—of β-adrenergic receptor Gi and Gs, and α1-adrenergic receptor Gq pathways, to the Ras/Raf/MAPKK/MAPK for the upregulation of transcription has been reviewed previously [43]. Furthermore, special consideration should be taken when interpreting results from in vitro, in vivo, and even in human in vivo, because of tissue-, and even species-specific differences in the mechanisms of regulation of tPA. Incidentally, evidence suggests that a glucagon-cAMP-PKA dependent mechanism blocks the upregulation of tPA in a traumatic brain injury model in pigs [44].

Similarly, if plasmatic levels of vWF were to be attributed exclusively to the β-adrenergic receptor/adenylate cyclase/cAMP signaling pathway, then use of atenolol should be accompanied with a decrease in the outcome, for which the opposite is shown in Table 3.2. In this case, while Table 3.1 displays results for a healthy group of subjects, vWF measurements were performed in a group of subjects with an underlying heart condition using a β1-adrenergic receptor selective blocker that exerts most of its
effects on the heart [45]. As such, it is plausible that, given that vWF levels are higher in hypertensive subjects [46] and they correlate with and even predict left ventricle hypertrophia (LVH) [47], use of 50 mg of atenolol a day is not sufficient to significantly lower baseline levels of vWF.

Although an empirical connection between β-adrenergic receptor agonism and increased activity of coagulation Factor V and decreased levels of PCGR are shown in Table 3.1, current state of the literature does not allow to backup this assertion directly and further research regarding this matter is required.

Intriguingly, long adrenergic receptor stimulation (Table 3.2) had an effect not much different than shorter stimulation (Table 3.1). Apparently, chronic, persistent catecholamine stimulation offers the same pro-coagulant net effect to subjects as acute dosing. However, longer adrenergic receptor stimulation was only described for individuals with a pre-existing chronic condition, which makes interpretation unclear. For technical and ethical reasons, only few studies have prospectively explored the effects of chronic catecholamine stimulation in human. However, accumulating results from observational studies univocally relate chronic mental stress to hypercoagulability, as seen in elderly Alzheimer caregivers [48–50], exhausted female school teachers [28], work-overwhelmed accountants [51], and workers with a negative relationship with their bosses [52], among many others. Nevertheless, it is without a doubt that chronic stress not only will influence levels of catecholamines, but also of cortisol [53], vasopressin [54], thyroid and hormones [55], among many others, and lack of control over the amount and progression of these endogenous stress hormones will always be an inherent source of confusion when attempting to elucidate the mechanisms underlying such effect. Thus, as observational studies fall short and prospective studies are technically and ethically challenging, in vivo studies allow for more accurate mechanistical observations. Yet, finding well suited animal experiments regarding the effect of adrenergic receptor-stimulation, or even chronic stress, on hemostasis or hypercoagulability from a mechanistical intracellular perspective is not an easy task. Besides the foreseeable inotropic effects (rapid elevation of systolic and diastolic blood pressure by approximately 40 mmHg with respect to baseline), continuous infusion of natural catecholamines in rats causes a significant increase in tissue weight and protein content in the aorta and the heart within 6 days of infusion [56]. Additionally, and considering catecholamine pharmacokinetics during chronic stress, it would also appear that not only the duration of the stimulus is important, but also the concentration of the hormone and the time-coordinated presence of α- or β-adrenergic receptors at the cell surface [57]. Consequently, for all these considerations and current limitations, the stepping stone is further experimentation.

3.5 Strengths and Limitations

In harmony with the scarce number of articles regarding the effects of adrenergic receptor stimulation on human hemostasis in the last 10 years, the last systematic review about this subject dates back to the year 2000 [58]. Thus, the present systematic review represents a unique updated appraisal of the observations and mechanisms gathered 22 years ago, now executed under the rigorous guidelines laid by the PRISMA 2020 statement [59]. Nevertheless, the fact that only 4 records met the inclusion criteria is, by all means, the foremost limitation to this study, and it embodies a reflection of how overlooked this field has been over the last decade. Far from discouraging our intentions for writing—and because experimental interventions were found sound and mostly unbiased, results assessed in the synthesis process were coherent, and outcomes were biologically plausible, notwithstanding the pathological baseline for subjects in Table 3.2—this scarcity of data highlights the need for further investigation at the basic/clinical level, turning the succinct nature of this systematic review into a calling for more research in the field, especially when addressing duration of the adrenergic receptor stimulus.
3.6 Conclusions

In sum, this systematic review shows that a prothrombotic anti-fibrinolytic state was induced after β-adrenergic receptor agonist stimulation and the opposite was induced after α₁-β-adrenergic receptor antagonist stimulation. Effect of the duration of adrenergic receptor stimulation offers inconclusive information that warrants further research.

Statements and Declarations

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Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Authors’ Contributions SG and DA extracted and analyzed the data. SG, CE, CR, JFS, and FS, collectively contributed to manuscript drafting. All authors read and approved the final manuscript.

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\(\alpha_1\)-Adrenergic Stimulation Increases Platelet Adhesion to Endothelial Cells Mediated by TRPC6

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**Abstract**

Stimulation of \(\alpha_1\)-adrenergic nervous system is increased during systemic inflammation and other pathological conditions with the consequent adrenergic receptors (ARs) activation. It has been reported that \(\alpha_1\)-stimulation contributes to coagulation since \(\alpha_1\)-AR blockers inhibit coagulation and its organic consequences. Also, coagulation induced by \(\alpha_1\)-AR stimulation can be greatly decreased using \(\alpha_1\)-AR blockers. In health, endothelial cells (ECs) perform anticoagulant actions at cellular and molecular level. However, during inflammation, ECs turn dysfunctional promoting a procoagulant state. Endothelium-dependent coagulation progresses at cellular and molecular levels, promoting endothelial acquisition of procoagulant properties to potentiate coagulation by means of prothrombotic and antifibrinolytic proteins expression increase in ECs releasing them to circulation, the thrombus formation is strengthened. Calcium signaling is a main feature of coagulation. Inhibition of ion channels involved in Ca\(^{2+}\) entry severely decreases coagulation. The transient receptor potential canonical 6 (TRPC6) is a non-selective Ca\(^{2+}\)-permeable ion channel. TRPC6 activity is induced by diacylglycerol, suggesting that is regulated by \(\alpha_1\)-ARs. Furthermore, \(\alpha_1\)-ARs stimulation elicits a TRPC-like current in rat mesenteric artery smooth muscle and mesangial cells. However, whether TRPC6 could promote an ECs-mediated platelet adhesion induced by \(\alpha_1\)-adrenergic stimulation is currently not known. Therefore, the aim of this study was to examine if the TRPC6 calcium channel mediates platelet adhesion induced by \(\alpha_1\)-adrenergic stimulation. Our results suggest that platelet adhesion to ECs is enhanced by the \(\alpha_1\)-adrenergic stimulation evoked by phenylephrine mediated by TRPC6 activity. We conclude that TRPC6 is a molecular determinant in platelet adhesion to ECs with implications in systemic inflammatory diseases treatment.
Keywords

Endothelial cells · α1-adrenergic receptor · Platelet adhesion · TRPC6 · Calcium

Abbreviations

ACD Citric acid dextrose buffer
ARs Adrenergic receptors
DAG Diacylglycerol
ECs Endothelial cells
FV Coagulation factor V
FVII Coagulation factor VII
FVIII Coagulation factor VIII
FX Coagulation factor X
FXa Activated coagulation factor X
FXI Coagulation factor XI
GPCR G protein-coupled receptors
GPIIIa Glycoprotein III a
GPIIb Glycoprotein II b
HUVEC Human umbilical vein endothelial cell
IP3 Inositol trisphosphate
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PIP2 Phosphatidylinositol 4,5-bisphosphate
PKC Protein kinase C
PLC Phospholipase C
PRP Platelet-rich plasma
SMC Smooth muscle cells
TAFI Thrombin activable fibrinolysis inhibitor
TF Tissue factor
TFPI Tissue factor pathway inhibitor
TM Thrombomodulin
tPA Tissue-type plasminogen activator
TRPC6 Transient receptor potential cation channel 6
TRPM7 Transient receptor potential melastatin channel 7
u-PA Plasminogen activator
vWF von Willebrand Factor

4.1 Introduction

Systemic inflammation, as well as other severe acute and chronic pathological states, considerably impair sympathetic nervous system function, resulting in elevated levels of endogenous catecholamines (norepinephrine and epinephrine) and consequent activation of adrenergic receptors (ARs) [1–11]. The adrenergic system is a complex interplay of neurohormonal mechanisms that regulate critical functions in the central nervous system such as sleep or autonomic outflow as well as peripheral functions such as cardiovascular control [12, 13]. This system is composed by the α- and β-adrenergic receptors, a ubiquitous group of glycoproteins that belong to the superfamily of G protein-coupled receptors (GPCR) and perform critical roles for the maintenance of vital functions such as the adaptation to environmental variations [14]. Particularly, the α-ARs are subdivided into α-1 and α-2 subtypes. The α1-AR subtype is mainly coupled to a Gq protein, which activates phospholipase C (PLC), cleaving phosphatidylinositol 4,5-bisphosphate (PIP2) to generate diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG/IP3 promote the activation of Ca2+ signaling pathways and also activates protein kinase C (PKC), which phosphorylates a number of proteins modifying their actions [15, 16]. Interestingly, it has been reported that α1 adrenergic stimulation contributes to coagulation since the use of the α1 adrenergic receptor blockers inhibited coagulation [17–19] and its organic consequences [20, 21]. Concordantly, coagulation induced by α1 adrenergic receptor stimulation can be greatly decreased using α1 adrenoreceptor blockers, while full inhibition can be achieved using both α- and β-adrenergic receptor blockers or non-selective blockers [22–25]. Given that the use of adrenergic receptor blockers decreased coagulation during systemic inflammation, it is feasible
to hypothesize that adrenergic stimulation, rather than pro-inflammatory mediators, is a critical element in promoting coagulation, making adrenergic stimulation a main factor to investigate [22, 25–27].

Hemostasis is the integration of molecular mechanisms preventing bleeding at the site of an injury while preserve normal blood flow in the circulation [28]. When there is an injury, the exposed molecules in the damaged tissue activate platelets, triggering thrombus formation. This event prevents blood loss in a platelet-dependent coagulation (primary hemostasis) [29]. Secondary hemostasis by the contrary, requires the active contribution of endothelial cells (ECs) lining the inner face of blood vessels, as well as the polymerization of fibrin in an insoluble mesh formed by platelets and even another circulating cells such as red blood cells, neutrophils and monocytes [30]. In health, ECs inhibit coagulation by acting as anticoagulants at the molecular and cellular levels. However, during inflammation, ECs become dysfunctional carrying out abnormal functions, playing a critical role in regulating coagulation [31, 32]. In this environment, endothelial cells could gain procoagulant properties supporting increased coagulation in an endothelium-dependent manner. This finding highlighted the notion that adrenergic stimulation generated during inflammation might modify normal function of ECs, shifting from anticoagulant environment into an altered procoagulant state.

Endothelium-dependent coagulation progresses at cellular and molecular levels supported by the reduction of anticoagulant and profibrinolytic molecules as well as the enhanced release of pro-thrombotic and anti-fibrinolytic molecules into the circulation, intensifying pro-coagulant states [9, 33, 34]. Endothelial anticoagulant molecules include thrombomodulin (TM) and tissue factor pathway inhibitor (TFPI) [35, 36]; the profibrinolytic include tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) [37, 38], while the prothrombotic proteins include tissue factor (TF) [34, 39]; the anti-fibrinolytic proteins include plasminogen activator inhibitor type 1 (PAI-1) and thrombin-activable fibrinolysis inhibitor (TAFI) [40–42]. Similarly, platelet–endothelial interactions are critical in coagulation progression, given that the glycoprotein IIb/IIIa (GPIIb/IIIa) for example, is the receptor mediating the binding between platelets and ECs [43, 44] via endothelial proteins such as von Willebrand factor (vWF) [45, 46], as well as the expression of other adhesion molecules by ECs such as adhesion molecule p-Selectin [47–49] and the integrin avb3 [50, 51].

Calcium signaling is a hallmark of coagulation [52–55]. It is known that inhibition of ion channels involved in Ca$^{2+}$ entry severely decreases coagulation [56–61], as well as the a1-AR signal transduction pathway is involved in the activation of Ca$^{2+}$ signaling during inflammation [15, 16]. Thus, Ca$^{2+}$ entry appears as a crucial step linking adrenergic stimulation with coagulation. Interestingly, the transient receptor potential canonical 6 (TRPC6) is a non-selective Ca$^{2+}$-permeable ion channel, composed by five-fold more permeable to Ca$^{2+}$ than Na$^+$ [62, 63]. TRPC6 exhibits rectification with single-channel conductance of $\sim$ 35 pS [63, 64]. TRPC6 is expressed in several cell types, including ECs, exhibiting higher expression than its homologues TRPC3 and TRPC7 [22, 65]. A functional channel is constituted by four TRPC6 subunits, that can be associated with TRPC3 and TRPC7 to form heterotetrametric channels, although their significance is poorly understood [62, 66, 67]. Since the initial findings showing that TRPC6 activity is induced by DAG, the notion that TRPC6 is regulated by G-coupled a1-ARs emerged [64, 68]. Results showing TRPC6 activation in response to PIP$_2$ hydrolysis mediated by PLCb, g and e [64, 68, 69], and that TRPC6 membrane translocation is regulated by GPCR activation [70, 71], reinforced the idea that TRPC6 is regulated by a1 adrenergic receptors activation. The notion that TRPC6 activity is regulated by ARs stimulation was finally demonstrated with results showing that a1 adrenergic receptors stimulation elicits a TRPC-like current in rat mesenteric artery smooth muscle cells (SMC) [72]. This finding was confirmed a decade later reporting that TRPC6 activity is induced by a1-ARs stimulation in
mesangial cells, showing that a1-adrenergic stimulation activates TRPC6-mediated Ca\(^{2+}\) influx, which was abolished by PLC inhibition [73]. TRPC6 phosphorylation by PKC inhibits channel activity, indicating that PLC, unlike PKC activity, is required to activate TRPC6 [74]. Thus, TRPC6 is a Ca\(^{2+}\)-permeable ion channel regulated by a1-adrenergic stimulation that appears as a suitable candidate to mediate coagulation-related actions in ECs. However, this hypothesis has not been demonstrated so far.

Participation of TRPC6 in endothelium-dependent coagulation induced by adrenergic stimulation is a novel idea. For that reason, little information is available. However, some studies performed in platelets in the context of wound healing condition, give the notion that such idea is supported. It has been reported that TRPC6 and TRPM7 mediate Ca\(^{2+}\) entry in platelets [75–78], which is crucial for platelet aggregation [78–81]. TRPC6 deletion in mice results in extended bleeding and coagulation time [81], but the global deficiency makes it hard to interpret the specific underlying mechanism. Taken together, it is suggested that TRPC6 could promote an ECs-mediated platelet adhesion induced by a1-adrenergic stimulation. However, this hypothesis is currently not known. Therefore, the aim of this study was to examine if the TRPC6 calcium channel mediates platelet adhesion to endothelial cells induced by a1-adrenergic stimulation.

Our results showed that endothelial cells exposed to the a1 adrenergic agonist phenylephrine exhibited an increased platelet adhesion to endothelial cells monolayer. Concordantly, endothelial cells exposed to the a1 adrenergic antagonist terazosin, did not show change in platelet adhesion. Interestingly, endothelial cells exposed to the a1 adrenergic agonist phenylephrine in the presence of the pharmacological TRPC6 blocker BI-749327, showed a total inhibition of the platelet adhesion induced by phenylephrine challenge, while the treatment with the TRPC6 blocker BI-749327 was efficient to inhibit the integrin avb3 expression increase induced by phenylephrine. The expression of von Willebrand factor and P-selectin were unchanged when endothelial cells were exposed to either phenylephrine or BI-749327.

These results could be useful in understanding the underlying mechanism of platelet adhesion to endothelial cells turning away from the complexity that involves the study of adrenergic over stimulation in a pathological and inflammatory context and could have implications in the treatment of systemic inflammatory diseases or enhanced adrenergic stimulation conditions.

### 4.2 Methods

**Cell Culture**: Human umbilical vein endothelial cell (HUVEC)-derived endothelial cell line EA.hy926 was cultured in Dulbecco’s Modified Eagle Medium (DMEM-Low) with 10% fetal bovine serum (FBS), 4 mM l-glutamine, 1 g/L d-glucose, 100 μg/mL penicillin–streptomycin and 2.5 μg/mL amphotericin. Cells were grown in an incubator at 37 °C in a humidified atmosphere (95% air and 5% CO\(_2\)). All cell culture supplies were purchased from Sigma-Aldrich.

**Cell Viability Assays**: Human umbilical vein endothelial cell (HUVEC)-derived endothelial cell line EA.hy926 was cultured in Dulbecco’s Modified Eagle Medium (DMEM-Low) with 10% fetal bovine serum, 4 mM l-glutamine, 1 g/L d-glucose, 100 μg/mL penicillin–streptomycin and 2.5 μg/mL amphotericin. Cells were grown in an incubator at 37 °C in a humidified
atmosphere (95% air and 5% CO₂). Passage took place every 2–3 days. All cell culture supplies were purchased from Sigma-Aldrich, USA. Toxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay after 24 h of incubation with Phenylephrine, Terazosin and BI-749327. MTT is a yellow compound that, when reduced by active mitochondria, produces purple formazan crystals that can be measured spectrophotometrically. For this purpose, MTT (Sigma-Aldrich, USA) was dissolved in phosphate buffered saline (PBS) to a concentration of 5 mg/mL and further diluted in culture medium (1:10). Cells were incubated with this MTT-solution for 4 h in an incubator at 37 °C in a humidified atmosphere (95% air and 5% CO₂). Afterwards, 100 µL of isopropyl alcohol were added. To completely dissolve the formazan salts, plates were incubated for 10 min on a shaker and quantified by measuring absorbance at 570 nm with an ELISA microplate reader. Cell viability was calculated as percentage of surviving cells compared against cells vehicle-exposed (control condition).

Platelets to Endothelial Cells Adhesion Assay:

Endothelial cells were plated at a density of 2.5 × 10^4 cells in a 96-well plate, and past 24 h were pretreated with the selective α-1 receptor inhibitor Terazosin (200 nM), the calcium blocker BI-749327 (500 nM) or transfected with an siRNA anti TRPC6 (siRNA^{TRPC6}) or siRNA control (siRNA^{Non-targeting}). For molecular TRPC6 inhibition, cells were transfected with 5 nmol/L siRNA (Dharmacon, USA) using lipofectamine (Invitrogen) and Opti-MEM (Gibco) and according to manufacturer instructions the transfection media was replaced after 4 h. Experiments were performed 48 h after transfection. To start the assay, the α1-adrenergic agonist Phenylephrine, was added at a final concentration of 0.1 µM with fluorescent-labeled platelets (∼ 2.25 × 10^6 platelets per well, contained in 100 µL of minimal experimentation medium) for 18 h at 37 °C. Then, non-adherent platelets were washed three times with warm PBS and observed in the FLoid Cell Imaging Station (ThermoFisher Scientific, USA). This experiment was made by technical triplicate and six fields for each condition by replicate were analyzed.

The images obtained were processed in grayscale using a pixel analysis software designed for these experiments. Briefly, the mean size of a platelet unit was calculated in pixels. Then, the number of total platelets per image was calculated as the fluorescence intensity of each pixel, then were grouped into 5 grays scales to apply the intensity correction factor.

Platelet Isolation from Human Peripheral Blood: The whole blood was obtained from normal male volunteer donors who declared have not consumed drugs that could interfere with platelet adhesion as anti-inflammatories, anticoagulants or antiplatelet drugs at least ten days before the blood extraction. Blood was collected in a Vacutainer tube with sodium citrate as anticoagulant. For each experiment 12 mL were collected. The extracted blood was transferred to 15 mL conical tubes and centrifuged at 200 g for 30 min at room temperature. Once centrifuged, the upper portion of the plasma, corresponding to platelet-rich plasma (PRP), was transferred to a new tube, and HEP Buffer was added in a 1:1 ratio, mixing it by inversion three times. Subsequently, the samples were centrifuged at 400 g for 30 min at room temperature to recover the supernatant again. To the latter, citric acid dextrose buffer (ACD) was added in 1:10 proportion, and then was mixed by inversion three times, centrifuged for 15 min at 3000 g at room temperature, and the pellet was recovered and resuspended in HEPES Tyrode’s buffer free of MgCl₂. Subsequently, the isolated platelets were counted using a Neubauer chamber and suspended at a concentration of ∼ 2.25 × 10^6 platelets/mL. Then, platelets were stained with the fluorescent dye vibrant DiO (ThermoFisher Scientific, USA) for 15 min at 37 °C. Platelets

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were washed by centrifugation at 3000 g for 5 min at room temperature three times. The pellet was resuspended in DMEM-Low 1% FBS and 100 µL of medium containing ~ 2.25 \times 10^6 platelets was added to the endothelial cells’ co-culture.

Quantification and Analysis of Platelet Adhesion to Endothelial Cells: The images obtained were processed in grayscale using a pixel analysis software designed for these experiments. Briefly, the mean size of a platelet or a neutrophil unit was calculated in pixels. Then, the number of total platelets or total neutrophils per image was calculated as the fluorescence intensity of each pixel, then were grouped into 5 grays scales to apply the intensity correction factor.

Detection of vWF, P-Selectin and Integrin αvβ3 by Fluorescent Immunocytochemistry: Pre-cultured and pre-treated ECs were washed twice with PBS and fixed with 3.7% paraformaldehyde (PFA) for 30 min at room temperature before being permeabilized with 0.1% Triton X-100 in PBS for 30 min at RT and then blocked for 2 h at room temperature with 3% Bovin serum albumin (BSA) in PBS. Cells were washed again and incubated with the primary antibodies to detect endothelial vWF (Invitrogen, USA), P-Selectin (Invitrogen, USA) and Integrin αvβ3 (Bioss, USA). Then, cells were washed twice and incubated with the secondary antibodies. Samples were mounted with ProLong Glass antifade mounting medium with NucBlue (Invitrogen, USA). For the quantification of fluorescence, areas of interest were selected and subjected to analysis using the Image J software. Fluorescence quantification was normalized against control condition (vehicle-treated condition).

Statistical Analysis: Results are presented as mean ± standard deviation or mean ± 95% confidence interval (CI) for the relative risk. Differences were considered significant at \( p < 0.05 \). Statistical differences were assessed by Student’s t-test (Mann–Whitney type), one-way analysis of variance (one-way ANOVA) See the figure legends for the specific test used.

4.3 Results

4.3.1 Platelet Adhesion Is Mediated by the α-1 Adrenergic Receptor

Considering the association between α-1 signaling and coagulation \([10]\), we examined whether platelet adhesion to ECs can be induced by activation of the α-1 adrenergic receptor. To that end, we performed experiments in phenylephrine-treated human ECs cocultured with isolated human platelets, and platelet-to-EC adhesion was measured. ECs treated with phenylephrine for 18 h showed an ~ 2.5-fold increase in platelet adhesion (Fig. 4.1b, e, g) compared with vehicle-treated ECs (Fig. 4.1a, d, g). Importantly, in ECs preincubated for 1 h with terazosin, the selective blocker of adrenergic α-1 receptors, phenylephrine-induced platelet adhesion was completely abolished (Fig. 4.1c, f, g).

4.3.2 TRPC6 Activity Mediates Phenylephrine-Induced Platelet Adhesion to ECs

On the other hand, it has been reported that there is a relationship between the activation of the α-1 adrenergic receptor and the activity of ion channel TRPC6 \([73, 82]\). So, we set out to examine the role of TRPC6 in phenylephrine-induced platelet adhesion to ECs. For this, we used the selective blocker of TRPC6 activity BI-749327, and remarkably, ECs preincubated for 1 h with BI-749327 completely abolished the phenylephrine-induced platelet adhesion (Fig. 4.2d, h, i).

4.3.3 Phenylephrine, Terazosin and BI-749327 Do Not Show Cytotoxicity in ECs

To demonstrate that platelet adhesion was mediated by receptor α-1 and TRPC6 activity, the cytotoxicity of all drugs Phenylephrine, Terazosin and BI-749327 was evaluated in ECs.
Fig. 4.1 Stimulation of the α-1 adrenergic receptor induces platelet adhesion to endothelial cells. Representative images from vehicle (a–d), phenylephrine (b–e) and terazosin-treated ECs (c–f) exposed to saline solution and then cocultured with platelets for 18 h. Scale bar represents 100 μm. Platelets were stained by Vybrant Dio (green), and platelet adhesion was analyzed (g) (N = 5). Results were normalized against vehicle-exposed cells (control condition). Statistical differences were assessed by a one-way analysis of variance (ANOVA) followed by Tukey post hoc test. ***p < 0.001 and ****p < 0.0001. Results showed as mean ± SD.

Fig. 4.2 Activity of TRPC modulates phenylephrine-induced platelet adhesion to endothelial cells. Representative images from vehicle (a–e), phenylephrine (b–f), BI-749327 (c–g) and phenylephrine + BI-749327-treated ECs (d–h) and then cocultured with platelets for 18 h. Scale bar represents 100 μm. Platelets were stained by Vybrant Dio (green), and platelet adhesion was analyzed (i) (N = 5). Results were normalized against vehicle-exposed cells (control condition). Statistical differences were assessed by a one-way analysis of variance (ANOVA) followed by Tukey post hoc test. ***p < 0.001 and ****p < 0.0001. Results showed as mean ± SD.
This was evaluated by MTT assay, it was observed that Phenylephrine does not present cytotoxicity in ECs in the concentration ranging from 0.1 to 10 $\mu$M (Fig. 4.3a) at 24 h. On the other hand, Terazosin showed low cytotoxicity in ECs up to 0.250 $\mu$M ($\leq$30%) at 24 h (Fig. 4.3b). Finally, BI-749327 showed no cytotoxicity in ECs at any of the evaluated concentrations after 24 h of exposure (Fig. 4.3c).

4.3.4 TRPC6 Expression Mediates Phenylephrine-Induced Platelet Adhesion to ECs

Next, to unequivocally demonstrate that endothelial TRPC6 is required for platelet-to-EC adhesion, we applied a molecular biological experimental strategy to downregulate TRPC6 expression using a small interfering RNA (siRNA) against the human isoform of TRPC6 (siRNA$^{\text{TRPC6}}$). Thus, experiments were performed in phenylephrine-treated ECs transfected with siRNA$^{\text{TRPC6}}$ or siRNA$^{\text{Nontargeting}}$, and platelet adhesion was determined. The results showed that siRNA$^{\text{TRPC6}}$-transfected ECs exposed to phenylephrine (Fig. 4.4d, m, j), completely prevented the phenylephrine-induced platelet adhesion (Fig. 4.4d, j, m) compared with vehicle-treated ECs (Fig. 4.4a–g, m) or siRNA$^{\text{Nontargeting}}$-transfected phenylephrine-treated ECs (Fig. 4.4f, l, m).

4.3.5 TRPC6 Is Required for Endothelial Integrin $\alpha\nu\beta3$ Expression Induced by Phenylephrine, but not for P-Selectin and vWF

It is well known that endothelial P-selectin, Integrin $\alpha\nu\beta3$ and vWF are crucial proteins that promote platelet adhesion to ECs [50, 51, 83]. Thus, we studied whether TRPC6 is required for vWF, Integrin $\alpha\nu\beta3$ and P-selectin expression in phenylephrine-treated ECs. Phenylephrine-treated ECs did not show an increase in vWF (Fig. 4.5a), and P-selectin (Fig. 4.5b). Interestingly, a significant increase was seen in the expression of Integrin $\alpha\nu\beta3$ (Fig. 4.5c), compared with the vehicle-treated condition. The pharmacological inhibition of TRPC6 with BI-749327 showed a significant prevention of phenylephrine-induced expression of Integrin $\alpha\nu\beta3$ (Fig. 4.5c), but not in the expression of vWF (Fig. 4.4a) and P-selectin (Fig. 4.4b).
4.4 Discussion

This work investigates the role of endothelium in a purer environment, with aiming to elucidate what is the real contribution of some molecules in the context of a hyperadrenergic state in four different axes, \( \alpha_1 \) adrenergic hyperstimulation and persistency, the activity of an adrenergic-stimulated calcium channel, TRPC6; the presence of TRPC6 itself and the effects on phenotypical changes in endothelium mainly focused on the expression of proteins related to favor a procoagulant state.

Coagulopathies are common and severe complications in diverse acute and chronic
diseases such as liver disease [84], coronavirus disease 2019 [85], cardiovascular disease [86] or sepsis [87]. Although coagulation is a complex response generally linked to wound healing when endothelial layer is disrupted, during inflammation, usually occurs in presence of endothelium, favoring its dysfunction. Coagulation represents a convoluted cascade that involves both molecular activation of coagulation factors that are present in plasma and endothelial phenotype modification allowing a cell adhesion phenomenon that favors thrombus formation. As it was mentioned before, coagulation mechanisms could activate without wound or injury present as well driving to thrombus formation. A thrombus can be defined as a blood clot formed inside a blood vessel in situ that alters or disrupts the blood flow driving severe complications. Thrombus formation occludes vessels generating hypoperfusion with subsequent anoxia in tissues [88]. Laminar flux of blood flow changes to turbulent which impairs transport of molecules between blood vessels and organs [89, 90]. In addition, the proportion of perfused blood vessels is considerably decreased, generating organ dysfunction [91] with a concomitant elevated mortality [92]. Central organs such as lung, kidney and liver are compromised tending to lose their functions. Besides, during thrombus formation, the fibrinolytic process to degrade thrombi also increases generating enormous D-dimer products as clinical indicators of thrombi degradation [37, 38].

It is well known that a pathological condition is often accompanied by inflammation, an immune response that implies a series of physiological and physio-pathological effects, one of which is the hyperstimulation of the adrenergic system, which leads to persistent increase in circulating catecholamines, triggering responses that are not necessarily focused on homeostatic parameters compensation. Both acute and chronic inflammatory signals are well recognized as triggers of sympathetic activation and hyperadrenergic states linked to adverse health effects [93] including increased cardiovascular risk [94, 95], immune dysregulation in coronavirus disease 2019 [96], increased mortality risk in septic shock [97] and monocyte activation and monocyte-platelet aggregates formation in hypertension [98]. Similarly, during inflammatory states, the anticoagulant actions performed by the endothelium to preserve blood fluidity are severely altered [99, 100], given cumulative evidence describing procoagulant states driving shortened coagulation and bleeding time, as well as the decrease in platelet count and reduced plasma levels of coagulation factors, a irrefutable indicator of consuming associated with thrombus formation processes [101, 102].

Diverse studies have shown a relation between α1 adrenergic stimulation and coagulation parameters, owing to the fact that several diseases requiring a treatment with adrenergic agonist or antagonist exhibit a direct or indirect effect on the coagulation system. For example, it has been well described that α-blockers drugs used in treatment to improve benign prostate hyperplasia symptoms as doxazosin and terazosin significantly increased coagulation time and bleeding time, suggesting that α1 blocking favors an anticoagulant state [20]. Another study found that the use of carvedilol in heart failure exhibited to reduction in D-dimer concentration and main platelet volume (an indicator of platelet activation) [103]. Despite of the above, these phenomena were studied in vivo, rising questions about the effective contribution of the endothelial tissue to the coagulation system changes. Endothelium, as being a main performer in hemostasis regulation, takes relevance in coagulopathies development, so the understanding of its behavior during an hyperadrenergic event appears to be an interesting way to find new biomolecules having an important role in endothelial dysfunction as calcium channels activated by adrenergic system.

The interaction between endothelium and platelets is critical for preserving hemostasis under physiological conditions [104]. Inflammation in its place promotes it loss and favors coagulation hyperactivation, exacerbating adverse outcomes particularly when combined with catecholamines over exposition [105] as evidenced by immortalized murine endothelial cells exposed to supraphysiological concentrations of catecholamines.
that lose their morphological integrity [106] and platelets when exposed to catecholamines, that significantly increased their count and size, both of which are associated with elevated activation, recruitment and aggregation [107].

Our results indicate that α1-adrenergic stimulation can individually elicit a significant procoagulative response. Regarding the contribution of α1 adrenoceptor, the presence of different types of adrenergic receptors and their density is a relevant factor driving signaling cascades in endothelium in front of a hyperadrenergic state. There are some investigations about this in other endothelial cells, as in vitro cultured rat coronary microvascular endothelial cells, where it has been demonstrated a high presence of α1 adrenergic receptors, specially α1D isoform [108]. Despite there are not studies showing adrenergic receptor types expression in EA hy.926 cells, there are in cultures human umbilical vein endothelial cells (HUVEC), demonstrating a high presence of α1 adrenergic receptors. Interestingly, the expression of these receptors is dynamic, and could change in front of inflammatory responses [109]. Curiously, our results show to increase platelet adhesion and pro-adhesive protein expression in endothelial cells (Figs. 4.1 and 4.5), demonstrating that just the basal expression of α1 adrenergic receptor presence is enough to induce a procoagulant phenotype, but that could be a clue in the explanation of why in systemic inflammatory diseases endothelial dysfunction can be exacerbated. Also, adrenergic receptors present another way to be regulated. Catecholamines triggers short-term adrenergic receptors internalization which is suggested as a compensatory mechanism to limit unfavorable adrenergic effects [110]. However, our findings showed that adrenergic receptors internalization does not interfere with platelet adhesion progression. This is probably because α1 adrenergic receptor subtypes present differential time dependent mechanisms of distribution, internalization and signaling following stimulation [111], linked with long-term responses such as protein expression [112–114].

The contribution of TRPC6 in platelet-mediated function and hemostatic regulation is well characterized. Previous studies demonstrated for example, that TRPC6 regulate receptor-operated calcium entry (ROCE) and cytosolic calcium concentration in platelets [78, 115] and TRPC6 deficient mice demonstrated increased bleeding and coagulation time attributed to deficient platelet function [81]. Whereas in endothelium, TRPC6 activity has been mainly linked to inflammation progression since promoted endothelial shape change after thrombin stimulation [116] and its colocalization with platelet/endothelial cell adhesion molecule-1 modulated leukocyte (PECAM) regulate transendothelial migration following histamine exposure [117].

PLC activity, DAG production, and enhanced calcium signaling attributed to effector proteins as calcium channels are all part of the canonical α1-ARs signal transduction pathway [118]. DAG directly activated TRPC6 ion channel [64, 119], and interestingly, it was shown that TRPC6 modulated the calcium influx necessary for mesangial cell proliferation following α1 adrenergic receptor stimulation with phenylephrine [73].

Our results demonstrated that endothelial TRPC6 regulate platelet adhesion to endothelial cells, showing for the first time the contribution of the α1-AR/TRPC6 axis in endothelial-mediated hemostasis regulation. It is vital to note that a physiological response to adrenergic stimulation is highly reliant on diverse factors such as adrenergic receptor stimulation duration as well as their density and diversity, therefore, investigating independents contributions is a complex issue and demands the system to be simplified. Factors as the expression and density of proteins and the expected effect might be extrapolated to other proteins outside ARs, such as calcium channels. TRPC6 is widely expressed in blood vessels from different territories, but the analysis of its density showed that is mainly expressed in tunica intima, where is located endothelium, and adventitia [120]. The above
reaffirms the hypothesis that TRPC6 could be an important contributor to coagulation potentiation in a hyperadrenergic context.

Coagulant state progresses as a consequence of endothelium activation, especially in arteries, but also in veins and capillaries [121, 122]. Through activation process, endothelial cells are activated expressing proteins described above to promote platelet adhesion to endothelium in the whole body, initiating and maintaining a great mass of thrombi formation [123, 124]. It has been previously demonstrated that the exacerbated expression of endothelial adhesion proteins is a key step in thrombus onset. When analyzed the expression of proteins induced by adrenergic stimulation, was interesting to note that despite the role of von Willebrand factor, P-selectin, and integrin αvβ3 in the amplification of coagulation, only integrin αvβ3 shows a significant expression change in these conditions.

To put in context the importance of these macromolecules studied here, it is important to highlight that von Willebrand factor is a glycoprotein selectively expressed in endothelial cells and megakaryocytes, thus it is contained inside platelets [125]. The cellular storages of this protein are Weibel–Palade Bodies in endothelium and α-granules in platelets, respectively. The release of von Willebrand factor from the storage vesicles can be triggered by several agonists such as thrombin, histamine and other inflammatory molecular signals. These have in common the augment in cytosolic calcium concentrations [126]. This factor has diverse functions, but mainly related to hemostasis regulation to coagulation. For example, it mediates platelet aggregation and adhesion to vascular endothelium [125], carries coagulation factor VIII allowing it to circulate stabilized and preventing its abnormal activation [127], among other functions. Regarding the life of this protein, von Willebrand factor mechanisms that induce its expression or drive its clearance remain obscure. The latest studies suggest von Willebrand factor complex clearance involves two phases in vivo, fast and slow [128], but our results do not are affected by physiologic clearance processes.

Meanwhile, P-selectin is a protein that is also expressed in both platelet and endothelial cells, and it is found inside the same storage vesicles mentioned above [129, 130]. P-selectin, as well as von Willebrand factor, is presented in two forms: soluble and integrated to plasma membrane [131, 132]. Soluble P-selectin is described to come mainly from platelets, and it is suspected to have its own physiological activity. Interestingly, endothelial cells show that P-selectin exposed in membrane is recycled back into the cell [133] and this could collaborate with an increase of half-life of this protein in endothelial cells. Moreover, P-selectin is not only an adhesion molecule. First, it has been demonstrated that its interaction with monocytes can induce tissue factor (TF) expression, mediating the adhesion of platelets to white blood cells, thus, collaborating with maintaining the local coagulation response after injury for hours. Second, P-selectin can transduce signals in cells, allowing the expression of some important immunological and pro-adhesive molecules such as chemokines and β2-integrin [134].

Finally, integrin αvβ3 is a cellular receptor that binds several ligands containing the arginine, glycine, aspartate (RGD) motif, such as both fibrin and fibrinogen, von Willebrand factor, thrombospondin, and collagen and laminin, proteins related to cell adhesion to extracellular matrix [135]. It has been described that integrin αvβ3 plays important roles on endothelium aside from just induce strong adhesion. Studies collect the importance of this protein in angiogenesis and endothelial cells survival [136]. Also, a study suggests that integrin αvβ3 plays an important role in coagulation binding to factor XVIII, a transglutaminase that collaborates with fibrin net cross-link [137], thus, stabilizing the strong clot. Interestingly, the study also demonstrated that some glycoproteins that are present in platelets surface also have the capacity to bind this coagulation factor, so integrin αvβ3 could collaborate to platelet adhesion to endothelium in an indirect manner [138].

It is therefore necessary to evaluate the mechanisms of storage and expression of the studied proteins since the stimulation time with adrenergic receptors can lead to differential responses such as
transient metabolic signaling or gene expression mechanisms that allow de novo synthesis. In terms of transitory signaling, changes in protein levels are predicted as a result of their release from storage vesicles such as Weibel–Palade Bodies. When findings of a long-term stimulation, as given here, are analyzed, it can be suspected that proteins were synthesized from the novo. However, it is vital to keep in mind that proteins synthesis and clearance duration might present differential statio-temporal patterns. For example, the mechanism by which von Willebrand factor is eliminated at a cellular level remains unclear. The data presented here suggest functional and phenotypical characteristics, but not the molecular mechanisms underlying these phenomena. Therefore, further studies could be helpful to elucidate transcriptional mechanisms are possibly involved in this hyperadrenergic state, may be providing some clues and improving the understanding about the regulation of α1β3 and the other analyzed proteins by the administered treatments.

Despite the role of α1-adrenergic receptor overstimulation in hemostasis (blood fluidity maintenance) regulation remains poorly elucidated, we can conclude that TRPC6 is crucial in the regulation of platelet adhesion to endothelial cells. Therefore, TRPC6 appears as a very important candidate in the regulation of hemostasis alterations induced by acute and chronic inflammation.

Statements and Declarations

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Disclosure of Interests All authors declare they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Physical Activity, Burnout, and Engagement in Latin American Students of Higher Education During the COVID-19 Pandemic

Andrea González, Oscar Achiardi, Martina Valencia, and Claudio Cabello-Verrugio

Abstract

The coronavirus-disease-2019 (COVID-19) pandemic has had a devastating physical and psychological impact on society, especially on students. In this study, we describe the levels of physical activity (Physical-Activity-Questionnaire-Short-Form (IPAQ-SF)), Burnout (School-Burnout-Inventory for students (SBI-U)) and engagement (Utrecht-Work-Engagement-Scale-9 items (UWES-9S)) in a cohort of Latin American higher education students during the COVID-19 pandemic in 2020. We also determined whether physical activity, Burnout, and engagement are related according to gender and area of study.

Self-reported data from 571 Latin American students (64.79% women, 34.15% men; average age 25.24 ± 5.52 years) were collected via an online survey questionnaire. Spearman correlation analyses evaluated the associations between physical activity, Burnout, and engagement. Comparative analyses by gender and field of study were also performed. The results showed no correlation or association in the linear regression between the IPAQ-SF and SBI-U scores or between the IPAQ-SF and the UWES-9S scores. By gender, men had higher IPAQ-SF scores (p < 0.05) and reported higher intensity physical activity than women, but women had higher SBI-U scores (p < 0.05). No difference was found between men and women according to the UWES-9S scores (p = 0.28). There was also no difference in IPAQ-SF scores (p = 0.29) regarding the field of study. Our results suggest that women perform less physical activity than men, which is consistent with higher Burnout. However, physical activity was not associated with Burnout or engagement overall, which indicates that it was insufficient to prevent emotional stress in Latin American higher education students during a pandemic.

Keywords

COVID-19 · Pandemic · Higher education · Students · Physical activity · Burnout · Engagement
### 5.1 Introduction

In December 2019, a new respiratory virus called severe acute respiratory syndrome caused by coronavirus type 2 (SARS-CoV-2) began to affect the world [1]. SARS-CoV-2, which belongs to the coronaviruses, leads to a cardiovascular, respiratory, gastrointestinal, muscular, and neurological disease named coronavirus disease 2019 (COVID-19). Four common types of coronaviruses (alpha, beta, gamma, delta, and omicron, among others) have been recognized, among which alpha and beta types are known to infect the respiratory tract in humans [2]. The mode of transmission of COVID-19 is through droplets produced by talking, sneezing, or coughing, or suspended droplets that remain in the environment [3]. According to data from the World Health Organization (WHO), the common symptoms of COVID-19 are fever, dry cough, and fatigue. Still, there could be other less frequent symptoms that may affect some patients, such as loss of taste or smell, nasal congestion, conjunctivitis (eye redness), throat pain, headache, muscle or joint pain, different types of skin rashes, nausea or vomiting, diarrhea, and chills or dizziness [4].

Being highly transmissible between humans has generated a global pandemic, which has claimed the lives of a considerable part of the population and continues its course to the present day [1]. Worldwide, as of October 25, 2022, the WHO has reported approximately 625,248,843 confirmed cases of COVID-19, including 6,562,281 deaths, which are constantly increasing [4].

The COVID-19 pandemic has proven to be a problematic situation that has affected physical and emotional health worldwide since 2019. Chile and other Latin American countries instituted a health emergency, and people were confined to their homes in 2020. Although the quarantine effectively restrained the pandemic, strict rules and isolation disrupted habits and adversely affected the physical and mental health of the population. Data on the COVID-19 pandemic are nonetheless emerging rapidly, with studies conducted on university students, the general population, healthcare workers, and people with mental disorders [5]. Taken together, these studies confirm the negative impact of the COVID-19 crisis on mental health, particularly regarding anxiety, stress, and depression. This fact was exacerbated among young adults who rely upon positive peer interactions for their general well-being [6]. According to UNESCO statistics, the most challenging moment was April 2020, when over 1484 million students of all education levels were affected, specifically 84.8% of the global total. In this context, college
education was no exception because, during the same month, 170 countries and communities had closed their higher education institutions, affecting over 220 million college students [7]. University students continued to attend classes but shifted to an online format, which halted face-to-face teaching. Thus, the higher education system changed its conventional academic activities towards teaching exclusively online.

This new setting forced them to migrate to a program called “Emergency Remote Teaching”, which involved using virtual media without adequate preparation [8–11]. According to Pérez-Villalobos et al. (2021), in Chile, many students do not have sufficient and/or proper infrastructure or connectivity from their homes. A similar situation is present in other Latin American countries. Moreover, students from families with lower economic incomes have more significant problems accessing the equipment and necessary connectivity and difficulty handling the technology. Together, these factors generate a gap that can paradoxically increase when universities implement new technological teaching resources without adequate support with software and hardware [8].

On the other hand, online teaching can interfere with students’ private lives, disrupting their daily routines and habits and negatively affecting their mental health [12–15]. Furthermore, lockdown, physical/spatial distancing, and other restrictions psychologically affect students and negatively influence their mental well-being, increasing anxiety, and stress levels, as observed in Chinese, French, and Spanish students [5, 9, 16–19]. For example, French university student respondents to a World Mental Health survey for university students completed questions concerning COVID-19 confinement. It showed they experienced increased anxiety as well as moderate to severe stress during confinement [19]. Additionally, a study carried out on Spanish university students whose objective was to design and validate an instrument to measure fear of COVID-19 obtained results that indicated that the level of fear of COVID-19 was moderate. In contrast, other authors carried out the same measurement on Iranian university students and the Bangladeshi population, resulting in high levels of fear of COVID-19 in both studies [20, 21]. Apparently, psychological responses vary according to culture and socio-demographic aspects [20, 21].

Under these new academic circumstances, and considering that college students constitute a particularly vulnerable population regarding mental health problems, it is probable that they will develop emotional disturbances, stress, a low sense of engagement (or degree of involvement) in their educational obligations, and emotional and/or physical fatigue—all typical features of burnout syndrome [19, 22, 23]. Burnout was first described in the 70s in the work context. Maslach defines it as “a psychological disorder syndrome arising as a prolonged response to interpersonal stressors at work,” and she describes three different types of Burnouts: Individual Burnout caused by personality factors such as perfectionism; Interpersonal Burnout caused by problematic relationships with colleagues; and Organizational Burnout caused by the inadequate organization (as extreme requests and deadlines) [24, 25]. Burnout syndrome is considered a psychological state that happens in normal individuals, where there is an inability to cope with the resulting emotional stress of prolonged exposure to stressful factors at work, which leads to occupying all the person's resources in it, leading to feelings of exhaustion and failure [26, 27]. Burnout is characterized by emotional exhaustion, cynicism, depersonalization, and reduced professional efficacy [4]. It is included in the 11th Revision of the International Classification of Diseases (ICD-11) as an “occupational phenomenon,” and World Health Organization defined Burnout as an “occupational official phenomenon” as it refers specifically to the occupational context [4]. Therefore, it should not be used in reference to other areas of life. This syndrome of emotional exhaustion, depersonalization, and reduced personal accomplishment can apply to all individuals engaged in work activities and other psychologically “work-like” activities, such as studying [28].

Like formal workers, university students can be exposed to risky conditions for the
development of Burnout characterized by concurrent, excessive, and inappropriate demands, chronically unresolved or inadequately resolved, which lead, over time, to significant psychosocial damage [29]. Student burnout is a psychological syndrome that manifests as exhaustion from the intense demands of studying, cynical attitudes toward study goals and learning, decreased performance, and feelings of incompetence [30]. The student has a feeling of not being able to give more of himself, an attitude of negative criticism, devaluation, loss of interest, importance, and value of studies, and growing doubts about his ability to carry them out [31]. Regarding academic learning, emotional exhaustion refers mainly to the inability to deal with problems encountered during the learning process, leading to negative emotions, Burnout, and depression. Cynicism is perceived as the development of a negative, pessimistic attitude and the feeling of decreased efficacy and academic achievement, manifesting as arriving late, leaving early, skipping classes altogether, and failing to complete academic tasks on time. Low professional effectiveness (the feeling of accomplishment) refers to students' lack of a sense of achievement when completing school requirements [30, 32, 33].

Student Burnout among university students can affect their physical and mental development, manifesting as a lessened feeling of engagement, drowsiness, fatigue, development of eating disorders, migraines, emotional instability, depression, illicit drug use, alcohol use, arterial hypertension, and even higher risk of myocardial infarction and suicidal thoughts [34–39]. These symptoms can also lead to the waste of educational resources, the decline of the students' learning ability, and the possibility of carrying these symptoms onward, which can have adverse effects on their work performance and personal lives [40, 41].

Another aspect that could be affected in university students with the non-face-to-face modality of classes during the pandemic and confinement is engagement. Engagement is a concept initially associated with work that is characterized by vigor, dedication, and absorption. However, it has also been applied to the activities carried out by students, defining it as academic engagement. The reasons for them are that from a psychological point of view, the activities carried out by students can also be considered as work since they are goal-directed, structured, and mandatory [42]. In engagement, vigor refers to a person's high levels of energy and mental resilience while studying, the willingness to put in the effort to study, and persistence when faced with difficult situations. Dedication refers to being fully involved in studies, finding meaning in them, and approaching them with enthusiasm, inspiration, pride, and challenge. Absorption refers to being wholly concentrated and absorbed in what is being studied, which makes time go by quickly and makes it difficult for the person to "detach" from the study [43]. Engagement is favorable for students since it is related to high levels of well-being, more significant commitment to their studies, and better academic performance [44]. It is currently known that engagement depends on the student's characteristics, such as personality, positive emotions, and personal resources [45]. Probably due to a lack of psychological support, the direct collaboration between peers, interaction with instructors, and community support occurs poor learning management decreasing motivation and engagement [46].

On the other hand, in a younger population, another possible adverse effect of a pandemic was the increase in unhealthy habits, such as the consumption of alcohol and tobacco, the adoption of a high-fat diet, the decrease in the intensity and quantity of physical activity, and an increase of sedentary time [6, 16, 17, 47–49]. In fact, it is known that during the pandemic, the adverse effects also affected people's lifestyles, with a decrease in the level and intensity of physical activity, as well as an increase in the consumption of less healthy food, an increase in sedentary behavior and increased consumption of alcohol and tobacco along with higher levels of anxiety between 18 and 34 years [6, 16, 17, 47]. Physical activity is a relevant aspect due to its well-known benefits for the general health of
individuals. It is defined by the World Health Organization (WHO) as “any bodily movement produced by skeletal muscles, with the consequent consumption of energy. Physical activity refers to any movement, even during leisure time, to move to certain places and from them, or as part of a person's job” [50]. During the pandemic, García Tascón et al. showed that the quantity and intensity of physical activity decreased in women and men, from cardiorespiratory and strength training (greater intensity) to flexibility and neuromotor exercise (lower intensity) [48]. Similarly, Castañeda-Babarro et al. showed that physical activity and intensity in healthy adults decreased during confinement and sedentary time increased, primarily in young people, students, and formerly very active men [51].

The relevance of these changes in physical activity during the pandemic is essential to understanding the adverse effects on physical health and possible impact on mental health. In this regard, the relationship between physical activity and a person’s psychological well-being, emotional state, mood changes, stress reduction, anxiety, and depression has been well documented [52, 53]. Researchers have attempted to determine the amount, type, and intensity of physical activity that might have optimal preventive effects on mental health. To date, results have indicated that regular physical training (strength and resistance)—independent of intensity—could be beneficial in reducing depression and anxiety symptoms [53–55]. Therefore, the reduction of students’ physical activity could negatively impact their mental health, especially with the concurrence of the pandemic, lockdown, and shift to online academic activities.

The COVID-19 pandemic changed life as we once knew it; the teaching–learning process was not unaffected. Students abruptly exchanged conventional face-to-face classes with remote classrooms to protect themselves and others. Unfortunately, this change in how classes are conducted has adversely affected young people’s social, emotional, and mental health and lifestyle habits, affecting college students’ academic performance. To study this phenomenon, we conducted research on physical activity, Burnout, and engagement in a population of Latin American higher education students during the COVID-19 pandemic. In addition, we determined whether physical activity, Burnout, and engagement were related to each other according to gender and area of study.

5.2 Methods

Different instruments were selected to assess the outcomes of interest, considering the availability of a validated Spanish version and the feasibility of self-administered questionnaires. For burnout and engagement, versions especially aimed at higher education students were selected.

Physical activity is defined by the World Health Organization (WHO) as “any bodily movement produced by skeletal muscles, with the consequent consumption of energy. Physical activity refers to any movement, even during leisure time, to move to certain places and from them, or as part of a person's job” [50].

Physical activity was assessed using the Spanish version of the International Physical Activity Questionnaire–Short Form (IPAQ-SF). This questionnaire was used for initial screening and research. The IPAQ-SF consists of four domains intended to measure levels of physical activity, estimated from the average time devoted to walking as well as activities of moderate to vigorous intensity during the previous week. Through four general questions, the IPAQ-SF collects relevant information about the time participants are physically active. The four general questions related to the time spent by the person in vigorous physical activity, moderate physical activity, walking, and sitting time, concerning the 7 days before its application. The scale categorizes vigorous physical activity as those that require strong physical exertion and cause the subject to breathe harder than normal; moderate physical activity such as those that need moderate physical effort and cause the subject to breathe something stronger than normal; walking physical activity, including walking around the house, to go from one place to another.
or any other walk that the subject performs for recreation, sport, exercise or pleasure and the time in which the subject has remained seated, either in his work, at home, studying and in his free time, which includes the time of visits, time sitting or lying down reading and/or watching television. In all cases, the considered physical activity must have been performed for a minimum of 10 continuous minutes within the last 7 days. The instrument uses as a measure for the activity the unit METs, which corresponds to a unit of measurement of the metabolic rate concerning the minimum oxygen consumption that the body needs to maintain its vital functions. Total physical activity was expressed in metabolic equivalent (MET) minutes per week, which was found by multiplying the number of days that a respective activity was performed by its average duration in minutes by a MET factor defined for each type of activity (e.g., walking = 3.3 MET, moderately intensive activity = 4.0 MET, and vigorously intensive activity = 8.0 [56, 57].

**Burnout syndrome** is defined as a psychological syndrome that manifests as exhaustion from the intense demands of studying, cynical attitudes towards study goals and learning, decreased performance, and feelings of incompetence. Initially, the MBI (Maslach Burnout Inventory) questionnaire, also known as the MBI-Human Services Survey (MBI-HSS), was used to investigate burnout syndrome in health workers. This survey had 22 items where the three characteristic variables of Burnout in health professionals were evaluated: exhaustion, depersonalization, and low professional efficacy. Exhaustion refers to that feeling of not being able to give more of oneself; depersonalization refers to having a cold attitude and distant treatment with patients, and low professional efficacy, refers to the feeling of not correctly executing professional functions and not being able to be competent when solving problems of the job function that arise. Later, Burnout began to be studied in other work areas, so the questionnaire became the MBI-General Survey (MBI-GS) that could be applied to people in any type of job. This questionnaire is more abbreviated and has 16 items where the dimension of exhaustion and low professional efficacy (which are part of the original questionnaire) are evaluated. The depersonalization or cold and distant attitude toward patients was changed for depersonalization in relation to work, a concept named as cynicism that corresponds to cold, indifferent, or distant attitudes towards work in general. Burnout in students was then evaluated, for which the MBI-GS was adapted to university students, obtaining the MBI-SS (Maslach Burnout Inventory-Student Survey) questionnaire, assuming that the academic activity of a student is equivalent to that of a formal worker. Currently, there is also the School-Burnout Inventory (SBI-U), an instrument validated and applied in various languages, including Spanish, which has 9 items. This questionnaire does not yet have normative values. Still, it allows for identifying the presence of Burnout syndrome in university students with acceptable reliability and is very similar to the MBI-SS questionnaire. In this research, Burnout was assessed using the Spanish version of the School Burnout Inventory adapted for college students (SBI-U), which consists of nine items graded on a Likert scale (1 = Completely disagree to 6 = Completely agree), separated into three domains (sub-scales) regarding exhaustion, cynicism, and inadequacy. The total score was achieved by adding all the scores together [58–61].

**Engagement** is a concept initially associated with work that is understood as a positive, satisfactory mental state characterized by vigor, dedication, and absorption. However, it has also been applied to the activities carried out by students, defining it as academic engagement. The reasons for them are that from a psychological point of view, the activities carried out by students can also be considered as work since they are goal-directed, structured, and mandatory. Academic engagement promotes learning, academic performance, and the well-being of the student, which is why it is so important to evaluate it. To evaluate engagement, a survey was initially created to assess engagement in students (UWES-S) that had 17 items and is known as the extended version. Then a short version of the
same scale was developed with 9 items, called UWES-S9. Regarding the information that needs to be obtained from the scale (extended or abbreviated), it is suggested that if you want to get differentiated information on the three dimensions of engagement, it is better to use a three-dimensional analysis, but if you wish to obtain information on the concept of engagement (not each of its components, it is preferable to use the total score. This research assessed engagement using the 9-item version of the Utrecht Work Engagement Scale for students (UWES-S-9).

Engagement was assessed using the 9-item version of the Utrecht Work Engagement Scale for students (UWES-S-9). This instrument uses nine items graded with a Likert scale (0 = Never to 6 = Always), separated into three domains regarding vigor, dedication, and absorption. For each domain, a score was achieved by averaging the scores of the items within that domain. A total score was achieved by adding the average scores [62, 63].

These three instruments were combined into a comprehensive survey (via surveymonkey.com) that included questions regarding general demographics, such as age, gender, and nationality. The survey was conducted following the Declaration of Helsinki and was approved by the ethics committee of the Universidad Andrés Bello in Santiago, Chile (Approval number 017/2020). The survey was piloted primarily by 34 undergraduate students to identify any problems with redaction or structure.

After corrections were made, the survey was distributed via e-mail and links on social media platforms that targeted higher education students from Latin America (native Spanish speakers). Students were required to be registered at any formal post-secondary educational institution (universities, technological institutes, art institutes, etc.). There was no discrimination regarding age, gender, or field of study. Participants gave informed consent to the research and their involvement before gaining access to the complete survey. Responses were collected for a total of 12 weeks.

After survey collection, the data were exported to a spreadsheet for codification and quality control. Then, the data were transferred to a statistical software package (GraphPad 9.3.1), and analyses were performed according to their distribution. Comparisons among genders were made for total scores for physical activity, Burnout, engagement, and the proportion of physical activity levels using the Mann–Whitney test. Correlations (using the Spearman test) and linear regressions were performed separately for men and women in terms of physical activity, Burnout, and engagement. Finally, comparisons among students from different areas of knowledge were made according to the ISCED fields of education and training (ISCED-F 2013), defined by UNESCO [64]. A p-value of < 0.05 was considered statistically significant.

5.3 Results

The participants in this research were students pursuing higher education in different fields of study. They were from various Latin American countries, including Argentina (n = 9; 1.57%), Bolivia (n = 1; 0.17%), Chile (n = 369; 64.62%), Colombia (n = 12; 2.10%), Costa Rica (n = 14; 2.45%), El Salvador (n = 149; 26.09%), Honduras (n = 6; 1.05%), Mexico (n = 7; 1.22%), Peru (n = 1; 0.17%), and Venezuela (n = 3; 0.52%). Table 5.1 shows the demographics of the survey participants, sorted by their field of education.

After data collection, we evaluated the participants’ physical activity levels during the COVID-19 pandemic by totaling their IPAQ scores and comparing them by gender group. As seen in Fig. 5.1a, men scored higher than women (p < 0.005). In addition, when the intensity levels of physical activity were analyzed, we found that men performed more activities of moderate to vigorous intensity than women (Fig. 5.1b). Next, we evaluated Burnout by comparing the participants’ SBI scores, grouped by field of study. Figure 5.2 shows that women had higher SBI scores than men (35.61 ± 0.49
and 32.78 ± 0.69, respectively) (p < 0.005). Then, using the UWES-ES, we evaluated the participants’ engagement with academic activities. As seen in Fig. 5.3, there was no significant difference between men and women (3.61 ± 1.34 and 3.76 ± 1.24; p = 0.2893). Finally, we determined that the IPAQ scores did not correlate with the SBI scores (r = −0.182, p = 0.01, respectively) or the UWES-ES scores (r = −0.167, p < 0.01). A linear regression analysis also showed no association between IPAQ scores and SBI scores (r² = 0.035, p < 0.01) or between IPAQ scores and UWES-ES scores (r² = 0.045, p < 0.01).

We then evaluated the students’ IPAQ scores when grouped by field of study (Health Science, Education, Technology, Social science, Basic science, and Administration & commerce). We found no significant differences between the students based on what they majored in (see Fig. 5.4). Figure 5.5 compares the SBI scores among the fields of study. Of note, technology students scored higher than social sciences students (p < 0.05), and health sciences students scored lower compared to both social sciences and education students (p < 0.05).

### 5.4 Discussion

We applied a survey to Chilean university students to determine the behavior of physical activity, Burnout, and engagement during the COVID-19 pandemic. We also determine if physical activity, Burnout, and engagement are related to each other according to gender and area of study.

Our results indicate that men perform more intense, more frequent physical activities than women, which is consistent with the last national health survey (ENS) conducted by the Chilean Ministry of Health (MINSAL) in 2016–2017, where it is shown that women have a higher percentage of a sedentary lifestyle than men (90% vs. 83.3%), a situation that is repeated when comparing the data from the ENS for the years 2003 and 2009–2010 [65]. These results are also like those found in other populations, where the percentage of physical inactivity is 8% higher in women than in men. Among the possible causes of this fact are low socioeconomic status and the gender difference itself, which negatively affects women and manifests in the form of low security and accessibility to physical activities that are not paid for (such as walking,

### Table 5.1  General characteristics of students according to different areas of knowledge

| Field of Study          | Health science (n=249) | Education (n=105) | Technology (n=42) | Social science (n=102) | Basic science (n=44) | Administration and commerce (n=29) | Total (n=571) |
|-------------------------|------------------------|-------------------|------------------|------------------------|---------------------|-----------------------------------|---------------|
| **Age (years)**         | 24 (14.1)              | 25.5 (0.7)        | 26.1 (4.2)       | 27.7 (3.5)             | 25 (0.7)            | 27.3 (2.8)                        | 25.24 (5.52)  |
| **Sex**                 |                        |                   |                  |                        |                     |                                   |               |
| Female (%)              | 171 (68.67)            | 53 (50.47)        | 14 (33.33)       | 82 (80.39)             | 32 (72.73)          | 18 (62.06)                        | 370 (64.79)   |
| Male (%)                | 76 (30.52)             | 50 (47.71)        | 27 (64.28)       | 20 (19.60)             | 11 (25)             | 11 (37.93)                        | 195 (34.15)   |
| Prefer not to say (%)   | 2 (0.80)               | 2 (1.90)          | 1 (2.38)         | 0 (0)                  | 1 (2.27)            | 0 (0)                             | 6 (1.05)      |

Data were reported as mean (±SD). Sex was reported as a percentage (%) of the total n of each group.
**Fig. 5.1**  
(a) Men’s and women’s physical activity measured in MET minutes per week, as determined by the IPAQ.  
(b) Men’s and women’s frequency and duration of moderately to vigorously intensive activity. Values are expressed as mean and standard error. Statistical significance is indicated by $p < 0.05$ with a 95% CI.

**Fig. 5.2** Burnout scores in men and women, as determined by the SBI. Values are expressed as mean and standard error. Statistical significance is indicated by $p < 0.05$ with a 95% CI.

**Fig. 5.3** Engagement scores in men and women, as determined by the UWES-ES. Values are expressed as mean and standard error. Statistical significance is indicated by $p < 0.05$ with a 95% CI.
On the other hand, traditional roles assigned to women, as well as a lack of social and community support, could also lead them to have less time for physical activities [67–73]. In this regard, women traditionally had to take care of housework, which in the pandemic became more noticeable, as well as those women who are mothers or caregivers had to dedicate full time to this type of unpaid work, which consumed the time they could have spent doing physical activity.

Additionally, our results show that women have higher SBI scores than men regarding Burnout, which is supported by and consistent with others mentioned in the literature where, in groups of university students during the COVID-19 pandemic, Burnout had a fundamental role in the development of symptoms of stress and depression [74, 75]. On the other hand, the studies showed that women presented more symptoms associated with depression, anxiety, and stress than men. Furthermore, symptoms worsened if a female student got sick with COVID-19, lived alone during quarantine, and/or had financial problems during that period [74–78]. Thus, Burnout played a fundamental role in the development of stress and depression in college students, especially in women during the COVID-19 pandemic. Some possible reasons for the gender difference in SBI scores could be that women have a greater need for social contact, a greater feeling of responsibility for the care and

Fig. 5.4 Differences in physical activity levels among students when grouped by field of study, determined by the IPAQ (MET-min/week). Values are expressed as mean and standard error. Statistical significance is indicated by p < 0.05 with a 95% CI

Fig. 5.5 Different burnout levels among students when grouped by field of study, as determined by the SBI. Values are expressed as mean and standard error. Statistical significance is indicated by p < 0.05 with a 95% CI

Fig. 5.6 Differences in engagement levels among students when grouped by field of study, as determined by the UWES-ES. Values are expressed as mean and standard error. Statistical significance is indicated by p < 0.05 with a 95% CI
well-being of family and friends, and less tolerance for changing situations [13, 75, 79]. Considering that college students have a greater predisposition than the general population to develop psychological disorders associated with Burnout and that women have an even greater propensity, the pandemic undoubtedly and negatively aggravated women’s mental health due to isolation, fear of contagion, information overload, and the stigma of being infected [74, 80–82].

In general, these unfavorable results for women could be due to the overload of unpaid work at home, added to the concern and fear of catching a virus about which little was known at the beginning of the pandemic. In addition, caring for sick people with COVID-19 in your family or close circle, and being concerned about those who have become seriously ill or have died during the pandemic, could also favor these results. Suppose we add to the above concerns for the livelihood of the home (which in many cases was lost), the full-time care of children and adolescents, and carrying out university studies. In that case, these situations may push anyone to the limit, mainly women.

Regarding engagement, although we did not find significant differences among the genders in this study, we know that as college students’ stress, anxiety, and depression levels rise, commitment to academics decreases, which results in more stress, eventually becoming a vicious circle of stressors—lowered engagement—more stress [79]. In relation to this result, it could be expected that since women have higher levels of stress and anxiety than men, they could have lower engagement scores than men in the context of the pandemic. However, in this research, we did not find differences according to sex. In this regard, it is essential to mention that the scores obtained in both men and women in this investigation are lower than those reported by other studies in Latin American students, where the general score is between 4.0 and 5.0 points, while in our results the scores are 3.6 and 3.7 in men and women, respectively. This decrease in the score compared to pre-pandemic studies could be due to the effect of confinement and the forced change to a virtual methodology, which could evenly influence male and female students in having less engagement [83].

Our analyses did not find any correlation or linear relationships among physical activity, Burnout, and engagement. This finding suggests that different factors determine physical activity levels and emotional health. Thus, we cannot assert that physical activity is a protective factor for the emotional health of Latin American higher education students. Our results differ from those reported by Deng and Wang. They claimed that typical and regular training of an adequate duration was associated with a lowered risk of developing mental disorders [84, 85]. A possible explanation for this discrepancy is that the students surveyed in Deng and Wang’s study quickly resumed physical education classes virtually once the online modality began; this situation probably did not occur in the Latin American study we analyzed. Furthermore, a technological gap could have affected the Latin American study if the situations had been similar. Another explanation could be related to the frequency (two or more days a week) and duration (more than 1 h) of physical activity reported by the authors (i.e., at least 120 min/week). The students in our sample averaged only 93.7 min/week of physical activity, and more than half reported low-level activity (data not shown).

The positive effects of physical activity on anxiety, depression, and other psychological disorders are known. However, outdoor and/or group physical activity (not in quarantine) may have a more positive effect than exercising alone and/or under lockdown, especially if contact with nature or socialization enhances motivation—performing physical activity in parks is associated with better levels of mental health. Thus, if chronic stress because of the pandemic reduces the willingness to perform any physical activity, depression symptoms arise, and a vicious circle forms [82, 86, 87].

When analyzing the data obtained by the field of study, we found that technology and health sciences students have a higher level of Burnout
and less engagement than students in social sciences and education. Our results agree concerning students in health sciences with the previous literature, which also found that those students show more stress-related illnesses, such as migraines, anxiety, and sleep disorders, than students from other programs [34–39]. A possible reason for this could be the small amount of time health students spend on physical activity [88, 89]. On the other hand, because they know more about health, they may feel greater responsibility for caring for their family, friends, and loved ones, a feeling that increases if they have children [90]. Furthermore, more knowledge of the possible results of a COVID-19 infection could be an additional stress factor. Other stressors include concerns about the labor market, the academic demands of the career, gender discrimination, criticism from patients and other medical personnel, peer comparisons, the gap between theory and practice, and concern about professional practice environment issues [91, 92].

The current literature shows no consensus regarding the results obtained from technology students, however. Some studies found high levels of Burnout and low levels of engagement, and other studies demonstrated lower levels of stress and depression in technology students compared to, for example, medicine or nursing students [93, 94]. On the other hand, reports from Naser (2021) and Vitasari (2010) showed that engineering students were likelier than others to suffer from depression and anxiety [95, 96]. Interestingly, if student demographics are considered, women studying technology who have economic problems, are married, and/or have children have more anxiety and depression significantly compared to men in the same field and other areas of study, which also coincides with the results of this research [95]. Possible explanations for the lack of consensus could be about the fear and concern that technology and engineering students feel about finding jobs after graduation, which may manifest as a lack of confidence, ambivalence, confusion, anxiety, depression, or lack of commitment [97].

5.5 Research Limitations

The results of this research should be interpreted with caution due to the limitations of the convenience sampling method, which could prevent the results from being generalized. Conducting a cross-sectional study could help identify causality between study variables. On the other hand, in future research, it would be interesting to analyze each area evaluated in the Burnout and engagement scales to identify subtle differences between women and men, as well as between the study area.

5.6 Conclusion

Considering this study’s findings, mental health issues need to gain significantly more attention in higher education institutions due to the pandemic-related increase in Burnout and decrease in engagement among college students [98] To better support students during a pandemic and/or quarantine, policymakers and school boards must consider findings from studies like this one when formulating policy recommendations and strategies [99] Furthermore, it is necessary to continue investigating college students’ physical and mental health, given the enormous change in teaching–learning methodologies that have been implemented during the COVID-19 pandemic—especially in Chile, where the digital and socioeconomic gap is significant.

Statements and Declarations

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Disclosure of Interests All authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval was granted by the ethics committee of the Universidad Andrés Bello in Santiago, Chile (Approval number 017/2020).

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Abstract

The immune system is the first defense against potentially dangerous chemicals, infections, and damaged cells. Interactions between immune cells and inflammatory mediators increase the coordinated activation of cross-talking signaling pathways, resulting in an acute response necessary to restore homeostasis but potentially detrimental if uncontrolled and prolonged. Plastic production exceeds million tons per year, becoming a global concern due to the stability of its constituent polymers, low density, which allows them to spread easily, and small size, which prevents proper removal by wastewater treatment plants, promoting environmental accumulation and increasing health threats. The interaction between plastic particles and the immune system is still being investigated, owing to growing evidence of increased risk not only for dietary intake due to its presence in food packaging, drinking water, and even fruits and vegetables, but also to emerging evidence of new intake pathways such as respiratory and cutaneous. We discuss in depth the impact of small plastic particles on the immune response across the body, with a focus on the nervous system and peripheral organs and tissues such as the gastrointestinal, respiratory, lymphatic, cardiovascular, and reproductive systems, as well as the involvement in increased susceptibility to worsening concomitant diseases and future perspectives in the exploration of potential therapeutics.

Keywords

Inflammation · Microplastics · Nanoplastics · Cytokynes · Oxidative stress

Abbreviations

ATP · Adenosine triphosphate
Bcl-2 · B-cell lymphoma 2
BEAS-2B · Bronchial epithelium transformed with Ad12-SV40 2B
6.1 Introduction

Plastic production exceeds million tons every year, becoming a global concern due to the stability of its constituent polymers, their low density which make them easy to spread and their small size which prevents proper removal by wastewater treatments plants, promoting accumulation and increasing health threats [1–3]. Rather of being chemically converted or mineralized, disposable plastics from food packages, gloves and others, are fragmentated into tiny particles known as microplastics (MPs) and nanoplastics (NPs) [4]. Although there is no consensus on how to classify them, the International Organization of Standardization determined particles as small as 5 mm as MPs and those small as 1 µM as NPs [5].

Studies attempting to elucidate biological absorption pathways, have shown interactions other than gastrointestinal, such as cutaneous and respiratory [6, 7]. Skin, for example, prevents MP and NP diffusion while allowing access through wounds or sweat glands [8]. The alveolar layer, by contrast, is thin enough to allow nanoparticles to permeate to the circulation [9]. Although it is unclear how MP and NP are taken by cells, current findings suggest that protein-plastic interactions might enhance cellular uptake [10], with endocytic mechanisms such as pinocytosis and phagocytosis, as suitable candidates [11].

Beyond its contribution in environmental concerns, the exploration of the interaction between plastic particles and human response is an ongoing issue considering its cytotoxic and genotoxic potential [12] as well as its ability to interact with and induce structural and functional protein changes [13]. Preliminary research on the effects of NPs in mice described that oral delivery was associated with plastic accumulation in
intestine, kidneys, and liver. Despite the fact that biochemical plasma measurements and histological samples showed no morphologic alterations, mice exposed to plastic particles exhibited an increase in inflammatory (levels of pro-inflammatory cytokines) and oxidative (reactive oxygen spices (ROS) production and superoxide dismutase (SOD) activity) status [14]. Similarly, after 5 weeks of oral MP and NP administration, an accumulation of plastic particles and aggregates was seen in intestinal lumen and spleen. Remarkably, the findings of this investigation revealed a sex-dependent response to plastic particles. While female animals do not store plastic in their reproductive system, male animals accumulated significant levels in their testes and seminaliferous tubules, which was associated with decreased testosterone production. Furthermore, only male splenocytes enhanced the expression of Interleukin 12 p35 (IL-12p35) and interleukin 23 (IL-23), amplifying the inflammatory response. Similarly, the anxiogenic response, locomotor activity, and gene expression in the brain indicated sex-dependent variations in response to plastic particles [15].

Moreover, 10 days of intranasal administration of MP and NP to rats resulted in weight loss, slimming, and decrease in cell layer on nasal mucosa, as well as elevated Transient receptor potential melastatin member 8 (TRPM8) expression, which was linked to respiratory inflammation. Although there were no morphological alterations in lung samples, histological abnormalities in kidney and liver were detected, suggesting that plastic passing for systemic circulation is the initial step in detrimental activities in peripheral organs [16]. Finally, after 28 of exposure to NPs, mice exhibited accumulation in stomach, intestine, testis, lungs, brain and spleen. While biochemical analysis revealed no significant changes, histological samples exhibited increased apoptosis and evident morphological damage. The liver presented increased infiltration of immune cells, vacuolization of hepatocytes and increased sinusoidal space; the lungs, showed alveolar wall thickness and interstitial fibrosis; the kidneys showed enhanced tubular atrophy, glomerulus alteration and immune cells infiltration; the intestine presented damaged epithelium and villi, epithelial permeability, crypt dysplasia and lymphocytes aggregation; the brain presented neuronal malformations in the cerebral cortex and finally, the testis, presented atrophy, immature germ cells accumulation in seminaliferous tubules and vacuolization in the same tissues [17].

6.2 Immune System

The immune system is the initial defense against potentially hazardous molecules, pathogens or damaged cells [18]. Mast cells, natural killer, granulocytes (neutrophils, eosinophils and basophils), dendritic cells and macrophages are immunological innate response cells [19], whereas inflammatory mediators include complement proteins, cytokines, nitric oxide and oxygen free radicals [20]. Interactions between immune cells with inflammatory mediators (released by immune and non-immune cells) promotes the coordinated activation of cross-talking signaling pathways, resulting in an acute response essential to restore homeostasis, which can be potentially harmful if uncontrolled and persistent [21].

The interplay between plastic particles and the immune system is still being explored, but interesting findings have already been reported (Table 6.1). The activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome in THP-1 cells (macrophages) by plastic particles was studied, revealing that amine-modified polystyrene particles activated NLRP3, whereas polyethylene terephthalate-, polycrylonitrile-, and nylon-derived particles increased IL-8 production in NLRP3-/- cells, demonstrating the pro-inflammatory properties of microplastics [22]. Similarly, the inflammatory response in human monocytes and monocyte-derived dendritic cells was significantly elevated by NP, particularly irregular NP exposure, as seen by increased pro-inflammatory cytokine release [23].

Furthermore, sulfate-modified NPs accumulated in cytoplasm lipid droplets in human
and murine macrophages and enhanced mitochondrial-mediated oxidative stress, promoting foam cell differentiation, a critical hallmark of atherosclerosis [24]. Similarly, NP boosted differentiation of murine macrophages into lipidic foam cells, which was associated with increased lipid droplet formation and accumulation in cytoplasm. Moreover, increased ROS production and lysosomal damage at high concentrations, suggesting that NPs alters lipid metabolism and promote lipotoxicity in macrophages [25]. After 3 h of exposure to heat-released particles, MP induced morphologic changes consistent with activation, whereas NP was significantly internalized and accumulated in the cytosol and lysosomes of macrophages, suppressing lysosomal activity associated with defective autophagy and increasing ROS generation, which could have direct implications in disease [26].

In human peripheral blood lymphocytes, NP produced 93% hemolysis and decreased cell

| Model                                                                 | Plastic type                      | Concentration | Time | Result                                                                 | References |
|----------------------------------------------------------------------|-----------------------------------|---------------|------|------------------------------------------------------------------------|------------|
| NLRP3-proficient and -deficient THP-1 cells                          | Micro and nanoplastics            | 4 mg/mL       | N/A  | NLRP3 [IL-8]                                                           | [22]       |
| Primary human monocytes and monocyte-derived dendritic cells         | Nanoplastic                       | 30–300 particles cell⁻¹ | N/A  | cytokine release                                                      | [23]       |
|                                                                      |                                   |               |      | pro-inflammatory response                                              |            |
| Human macrophages                                                    | Nanoplastic                       | 100ug/ml      | N/A  | acute mitochondrial oxidative stress                                    | [24]       |
|                                                                      |                                   |               |      | impaired lysosomal clearance                                           |            |
| RAW 264.7 macrophages                                                | Nanoplastic                       | 50ug/ml       | N/A  | lipotoxicity                                                           | [25]       |
|                                                                      |                                   |               |      | foam cell formation                                                    |            |
|                                                                      |                                   |               |      | atherosclerosis                                                        |            |
| Mouse macrophages                                                    | Micro and nanoplastic             | 3 h           |      | inflammation                                                           | [26]       |
|                                                                      |                                   |               |      | lysosomal activities of macrophages                                    |            |
| Human lymphocyte cells                                               | Polystyrene nanoplastics          | 2000 µg/mL, 1000 µg/mL, and 500 µg/mL | N/A  | chromosomal aberrations, such as chromosomal breaks and dicentric chromosomes
|                                                                      |                                   |               |      | nucleoplasmic bridge (NBP) formation and nuclear budding (NBUD)        | [27]       |
|                                                                      |                                   |               |      | frequency of mitotic index (MI)                                        |            |
|                                                                      |                                   |               |      | micronuclei (MN) formation and cytostasis%                             |            |
|                                                                      |                                   |               |      | nuclear division index (NDI)                                           |            |
|                                                                      |                                   |               |      | oxidative stress-mediated cytotoxicity, DNA damage, and genomic instabilities |            |
| Immortalized human lymphocytes                                        | Nanoparticles                      | N/A           | N/A  | oxidative stress, apoptosis and mortality                               | [28]       |
|                                                                      |                                   |               |      | cells’ Young Modulus                                                   |            |
| Mice neutrophils                                                     | Polystyrene nanoplastics          | N/A           | N/A  | NET formation, with involvement of reactive oxygen species, peptidyl arginine deiminase 4 (PAD4), and neutrophil elastasePAD4 | [29]       |

*N/A: Not available*
viability (by close to 60%). Similarly, NP administration increased chromosomal damage and genomic instability markers such as mitotic index, micronuclei frequency, cytostasis, chromosomal breaks, chromosomal rings, and dicentric chromosomes, affecting cellular proliferation as evidenced by the nuclear division index [27], and further studies in immortalized lymphocytes confirmed the cytotoxic effects of NPs [28]. Finally, NP exposure caused the release of neutrophil extracellular traps in murine neutrophils via synergistic mechanisms involving increased calcium signaling, ROS and elastase production, and peptidyl arginine deiminase 4 overexpression, resulting in histone citrullination and chromatin depolymerization [29].

6.3 Nervous System

The nervous system formed by a complex neuronal and glial network [30], allowing organisms attend self or external demands to preserve vital functions [31]. The inflammation in nervous system also known as neuroinflammation, is a common response in acute or chronic brain or peripheral diseases, being linked to impaired cognitive function or neurodegenerative diseases such as Alzheimer, Parkinson, amyotrophic lateral sclerosis, among others [32]. So, deciphering harmful potential of plastics and possible adverse effects in brain-mediated functions is a relevant issue (Table 6.2).

The effect of NP in nerve injury and subsequent learning impairment was also evaluated in mice exposed chronically to NP (6 months). The results showed that NP promoted detrimental effects in cognitive function and increased ROS and deoxyribonucleic acid (DNA) damage dose-dependently. Further experiments indicated that NP acted in synaptic terminals affecting close to 96 messenger ribonucleic acid (mRNA) and a complex network of competing endogenous RNA (ceRNA) involving 27 circular RNAs, 19 miRNAs and 35 mRNAs associated to synaptic alteration [33].

Moreover, in mice was demonstrated that 28-day exposure to NP induced neurodegeneration like Parkinson disease mediated by mitochondrial dysfunction associated-energetic metabolism impairment in neuronal cells, in excitatory neurons. Similarly, was found increased pro-inflammatory status in astrocytes and microglia and proteostasis alterations and synaptic dysfunction in astrocytes, oligodendrocytes and endotheliocytes [34].

In zebrafish, aminated-NPs accumulated in gastrointestinal tract and brain, affected development as evidenced the reduction of spontaneous movement, heartbeat, hatching, as well brain cell apoptosis promoting neurobehavioral damage through NMDA2B receptor interaction [35]. Similarly, NPs accumulated in gonads, intestine, liver and brain, impaired lipid and energy metabolism and increase oxidative stress in a size- and shape-dependent fashion. Interestingly, NPs also promoted neurobehavioral impairment as evidenced the altered locomotion, aggressiveness and dysregulated circadian rhythm mediated by toxic neurotransmitter accumulation [36].

In HT22 hippocampal neuronal cells exposed to MP and NP was observed increased attachment to cell surface and internalization with perinuclear localization independent of particles size. Interestingly, the smallest particles need to get clustered for effective intercellular trafficking being trapped in lysosomal structures, while MP were distributed in cytoplasm. Showing differential cellular distribution suggesting differential uptake mechanisms. Interestingly, results showed that a difference of other studies reporting that only NP promoted ROS, in this experimental setting, MP were also able to promote ROS increased after transient exposure (1 h) and, after long-term exposure (24 h), only NP promoted elevation in ROS production and importantly, these particles were mainly involved in cell cycle alterations and cell viability reduction. Authors hypothesize that NP promoted oxidative damage to macromolecules as DNA, lipids and proteins through mitochondrial mediated mechanisms or due intrinsic reactivity properties of NP [37].

In mice, NP administered for 7 days permeated blood brain barrier and accumulated in brain. The neurotoxicity and cerebral injury
| Model                  | Plastic type                        | Concentration               | Time                | Result                                                                                                    | References |
|-----------------------|-------------------------------------|-----------------------------|---------------------|-----------------------------------------------------------------------------------------------------------|------------|
| **Mice**              | Polystyrene nanoplastics            | 0, 10, 25, 50 mg/kg         | 6 months           | ↑ ROS levels and DNA damage ↓ injury in the synaptic function                                              | [33]       |
| **C57BL/6 J mice**    | Polystyrene nanoplastics            | 50 nm, 0.25–250 mg/kg       | 28 days            | ↑ Parkinson’s disease (PD)-like neurodegeneration ↓ dysfunction in all brain cells, and especially in excitatory neurons, ↑ inflammatory turbulence in astrocytes and microglia, dysfunction of proteostasis and synaptic-function regulation in astrocytes, oligodendrocytes, and endotheliocytes ↓ adenosine triphosphate (ATP) content and expression levels of ATP-associated genes and proteins | [34]       |
| **Zebrafish**         | Polystyrene nanoplastic             | 30 and 50 mg/L              | N/A                | ↑ stronger developmental toxicity (decreased spontaneous movement, heartbeat, hatching rate, and length) ↑ cell apoptosis in the brain ↑ neurobehavioral impairment ↑ levels of glycine, cysteine, glutathione, and glutamic acid ↑ levels of spermine, spermidine, and tyramine | [35]       |
| **Zebrafish**         | Nanoplastic                         | 0.5 ppm, 1.5 ppm, and 5 ppm | 7 days, 30 days and 7 weeks | ↑ disturbance of lipid and energy metabolism ↓ oxidative stress and tissue accumulation ↑ behavior alterations in their locomotion activity, aggressiveness, shoal formation, and predator avoidance behavior Affect fish reproductive function | [36]       |
| Mouse hippocampal neuronal HT22 cells | Micro and nanoparticles | 5, 25 and 75 µg/mL | 1 h and 24 h | ↑ ROS ↑ cytotoxicity ↑ neural cells | [37]     |
| Mice and immortalized human cerebral microvascular endothelial cell (hCMEC/D3) | Polystyrene nanoplastics | 0.5–50 mg/kg | 7 days | ↑ permeability of BBB ↑ reactive oxygen species (ROS) ↑ nuclear factor kappa-B (NF-κB) ↑ tumor necrosis factors α (TNF-α) secretion ↑ necroptosis of hCMEC/D3 cells ↓ transendothelial electrical resistance (TEER) | [38]     |

(continued)
elicited by plastic particles was mediated by microglial cells, which showed increased activation that promoted neuronal damage. In addition, in vitro explorations in microvascular cerebral endothelial cells showed increased internalization of NP, ROS and tumor necrosis alpha (TNF-\(\alpha\)) production, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-\(\kappa\)B) activation, necroptosis and tight junction disruption, explaining the crossing of plastic particles to brain [38]. Interestingly, NP aerosols has also shown hazard effects in brain since inhaled nanoparticles accumulated in brain and trigger neuronal toxicity and neuroinflammation associated to abnormal animal behavior [39]. Further experiments in mice exposed to NPs in drinking water for 180 consecutive days corroborated these findings through the observation of plastic accumulation in brain, disruption of blood–brain barrier, increased dendritic spine density and hippocampal inflammation concentration-dependent. These alterations promoted cognitive and memory impairment [40].

| Model                  | Plastic type          | Concentration | Time    | Result                                                                                                                                                                                                 | References |
|------------------------|-----------------------|---------------|---------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Mice                   | Nanoplastics          | N/A           | N/A     | ↑ deposit in the brain of mice via aerosol inhalation. ↑ fewer activities in comparison to those inhaling water droplets ↑ neurotoxicity                                                                 | [39]       |
| BALB/c Mice            | Polystyrene microplastics | 100 µg/L and 1000 µg/L PS-MPs | 180 days | ↑ exhibited disruption of the blood–brain barrier, ↑ level of dendritic spine density, and an inflammatory response in the hippocampus ↑ cognitive and memory deficits                                                                 | [40]       |

*N/A: Not available

6 Small Plastics, Big Inflammatory Problems

6.4 Respiratory System

Anatomically, respiratory system is composed by nose, oropharynx, larynx, trachea, bronchi, bronchioles and lungs. Although the lungs are the most studied and even considered the main and functional structure from respiratory system by facilitate gas exchange between inhaled air and blood [41], external components and upper airway has an essential role for filter harmful particles on air [42]. In front to irritant particles, the inflammatory response in airway is natural to initiate healing. However, this local inflammation is usually associated to airway remodeling and parenchymal destruction as same has been observed in acute respiratory distress, chronic obstructive pulmonary disease, asthma or cystic fibrosis [43].

Growing evidence about airborne plastics has showed their potential to penetrate and damage airways (Table 6.3). The analysis of 20 post-mortem lung samples, showed the presence of 33 polymeric particles, presenting the inhalation as another entry mechanism [44]. To clarify the effect of plastics in airways, a preliminary approach performed in A549, as an in vitro model of lung cells, evaluate the effect of MP and NP ranging 2 µM to 80 nm and the authors described that NP promote strong cellular damage compared to MP, affecting cell viability [45]. Complementary studies performed in the same cellular line evaluated the role of NP in lung fibrosis and results showed that NP, specially the positively charged, promoted alterations associated to fibrosis development including increased
migration and epithelial-to-mesenchymal transition, ROS, NADPH4 expression, mitochondrial dysfunction evidenced in membrane potential alterations and altered energetic metabolism and endoplasmic reticulum stress [46]. Finally, experiments performed in bronchial epithelium transformed with Ad12-SV40 2B (BEAS-2B) and human pulmonary alveolar epithelial cells (HPAEpiC) showed that NP exposure dose-dependently reduce cell viability, altered oxidative equilibrium and tight junctions, which could be associated to lung damage [47].

Among the mechanisms associated to the increased response to microplastics, long non-coding RNA and circular RNA emerged as targets since Sprague–Dawley rats instilled with MPs by three days showed increased deposits in lungs, alveolar and bronchial epithelium destruction, upregulation of pro-inflammatory cytokines such as interleukin 6 (IL-6), TNF-α and interleukin 1β (IL-1β) and significant increase in non-coding RNA XLOC_031479, circular RNA 014924 and circular RNA 006603 and the downregulated expressions of long

| Model                  | Plastic type                      | Concentration | Time   | Result                                                                 | References |
|------------------------|-----------------------------------|---------------|--------|------------------------------------------------------------------------|------------|
| Human lung tissues     | Airborne microplastics            | N/A           | N/A    | ↑ Deleterious health outcomes in respiratory system following inhalation | [44]       |
| A549 Cells             | Polystyrene Micro- and Nanoplastics | 5, 25, 100, 200ug/mL | 24 h   | ↑ cytotoxicity and genotoxicity  ↓ Cell viability and the induction of micronuclei (MN) | [45]       |
| A549 cells             | Polystyrene nanoplastic           | N/A           | N/A    | ↑ migration and EMT markers   ↑ up-regulation of reactive oxygen species (ROS) and NADPH oxidase 4 (NOX4), an ROS generator located in the mitochondria and endoplasmic reticulum (ER)  ↑ mitochondrial dysfunction as   ↑ ER stress as indicated by the up-regulated ER stress markers | [46]       |
| Human lung epithelial cells | Polystyrene nanoplastic       | 7.5, 15 and 30 μg/cm² | N/A    | ↑ cell viability 770 genes in the 7.5 μg/cm² group and 1951 genes in the 30 μg/cm² group were distinctly ↑ inflammatory effects, and triggered apoptotic pathways to cause cell death ↑ transepithelial electrical resistance by depleting tight junctional proteins ↑ matrix metallopeptidase and Surfactant protein A levels | [47]       |
| Rat                    | Polystyrene Microplastics and Nanoplastics | 100 nm (100 mg/10 ml), 500 nm (100 mg/10 ml), 1 μm (100 mg/10 ml) and 2.5 μm (100 mg/10 ml) | 3 days | Increased deposits in lungs, alveolar and bronchial epithelium destruction, upregulation of pro-inflammatory cytokines such as interleukin 6 (IL-6), TNF-α and interleukin 1β (IL-1β) and significant increase in non-coding RNA XLOC_031479, circular RNA 014924 and circular RNA 006603 | [48]       |

*N/A: Not available*
non-coding RNA XLOC_014188 and circular RNA003982, providing new insights of the genetic regulation exerted by MPs [48].

### 6.5 Circulatory System

Circulatory system comprises cardiovascular system and lymphatic system [49]. The first, the cardiovascular, consist of heart and vascular system (arteries, veins and capillaries) covering all the body and allowing proper nutrients and oxygen supply to all organ, tissues and cells [50]. During local or systemic inflammatory response, circulatory system and specially microcirculation, suffer structural (remodeling) and functional changes, increasing the reactivity of microvascular cells such as endothelial cells, pericytes and smooth muscle cells and circulating blood cells as platelets and red blood cells (RBCs), orchestrating thrombogenic responses, often life-threatening, in face to unregulated inflammatory events [51, 52] (Table 6.4).

NP, particularly amine-modified promoted structural and functional modifications in freshly isolated RBCs after its uptake, increasing aggregation, hemolysis, generation of microvesicles and adhesion to endothelial cells, involving calcium upregulation, and depletion of intracellular adenosine triphosphate (ATP) and glutathione (GSH), increased adhesion. Further, in vivo experiments performed in rats demonstrated that murine RBC presented similar response after exposure to plastic particles, increasing hemolytic activity as in human-derived cells. Interestingly, in vivo was observed the externalization of the pro-coagulant molecule phosphatidylserine and thrombin generation, promoting rapidly venous thrombus formation in rats [53].

In zebrafish embryos, MP and NP promoted pathological alterations of caudal vein plexus, impairing caudal tissues development and peripheral microcirculation disturbances [54]. Similarly, NP exposure promoted pericardial edema, decreased cardiac output, blood flow velocity, gastrointestinal angiogenesis, disturbed cardiovascular development, increased endothelial dysfunction, neutrophil recruitment, red blood cells aggregation and enhanced thrombosis formation in tail vein from zebrafish embryos [55].

The exposition of endothelial cells to anionic NP increased ROS production, autophagy via PI3K/AKT pathway, apoptosis mediated by Bcl-2 downregulation. Also was observed a notable internalization of NP and binding to vascular endothelial cadherin (VE-cadherin) unaffected transcriptional or post-translational expression but promoting disruption of adherent junctions and subsequent endothelial leakiness. Interestingly, the block of endocytosis does not prevent endothelial leakiness but was observed that NP promoted Src activation, and its pharmacological inhibition prevented VE-cadherin phosphorylation and gaps formation. Further in vivo exploration of mice injected with NP presented signs of vascular leak in brain, liver, spleen, lungs, kidney, and diaphragm [56]. Similarly, human umbilical vein endothelial cells (HUVECs) exposed to plastic particles, showed increased adhesion of the biggest particles, whereas the smallest were taken up and formed aggregates in the cytoplasm. Interestingly, long-term exposure of big particles for 48 h enhanced lactate dehydrogenase (LDH) production, autophagy and autophagosome formation, showing a that internalization and autophagy are size- and time-dependent in endothelial cells [57]. Conversely, HUVEC exposition to MPs and NPs showed no significant increase in inflammation, autophagy, ROS, LDH release and adhesion proteins expression. Only following 48 h of exposition a decrease in cell viability was observed [58].

In porcine coronary artery endothelial cells, the exposition to NP promoted a premature senescent associated phenotype featured by increased β-galactosidase activity, increased p53, p21 and p16 levels promoting reduced proliferation. Moreover, reduced eNOS activity impairing vasorelaxation, increased ROS production through exacerbated NOX (NADPH oxidase) activity and decreased sirt1 expression. Importantly, the use of antioxidant molecules prevents endothelial senescent phenotype and oxidative damage [59].
| Model                                      | Plastic type                  | Concentration                  | Time  | Result                                                                                           | References |
|--------------------------------------------|-------------------------------|--------------------------------|-------|-------------------------------------------------------------------------------------------------|------------|
| Human red blood cells Rats                 | Polystyrene nanoplastic       | 100 µg/mL and 500 µg/mL        | 3 h   | Morphological changes of RBCs by PS-NPs PS-NPs induced the externalization of phosphatidylserine, generation of microvesicles in RBCs, and perturbations in the intracellular microenvironment. ↑ activity of scramblases responsible for phospholipid translocation in RBCs ↑ thrombus formation | [53]       |
| Zebrafish embryos                          | Microplastics and nanoplastics | N/A                            | N/A   | ↑ mortality rate caused pathological changes of caudal vein plexus inhibition of intact growth of zebrafish embryos. ↓ Peripheral microcirculation at caudal region | [54]       |
| Zebrafish embryos                          | Polyethylene nanoplastics     | 50 µg/mL                        | N/A   | ↑ pericardial edema ↓ the cardiac output (CO) and blood flow velocity ↑ thrombosis ↑ Reactive oxidative stress (ROS) and systemic inflammation ↑ neutrophils recruiting in the tail vein | [55]       |
| HUVECs and Swiss mice                      | Nanoplastic                   | 0.05, 0.1, 0.25 and 0.5 mg/mL   | 1, 3 or 6 h | ↑ disrupted the vascular endothelial cadherin junctions ↑ vasculature permeability | [56]       |
| Human umbilical vein endothelial cells     | Polystyrene nanoplastic       | 25 µg/mL                        | 48 h  | ↑ lactate dehydrogenase release from HUVECs ↑ cell membrane damage, ↑ autophagy initiation and autophagosome formation | [57]       |
| Human umbilical vein endothelial cells     | Polystyrene microplastic      | 5, 10, 25 and 100 µg/mL         | 48 h  | ↓ cell viability                                                                                   | [58]       |
| Porcine coronary artery endothelial cell   | Polystyrene nanoplastic       | 0.1, 1, and 10 µg/mL            | 6 h   | ↑ senescence markers, senescence-associated β-galactosidase activity, and p53, p21, and p16 protein expression ↓ proliferation ↓ endothelial nitric oxide synthase (eNOS) expression ↑ reactive oxygen species formation in ECs, and increased oxidative stress levels were associated with the induction of NADPH oxidaes expression, ↑ Sirt1 expression | [59]       |

(continued)
The lymphatic system on the other hand, has traditionally been known as a complement of cardiovascular system or a simple drainage system since consistent in a unilateral transit pathway from interstice to venous territory [60, 61]. Components of the lymphatic system include the primary lymphoid organs, composed by thymus and bone marrow; the secondary lymphoid organs, including lymph nodes, spleen and Peyer’s patches [62]; and finally, the tertiary lymphoid organs, also known as inducible lymphoid organs, tertiary lymphoid tissues or ectopic lymphoid organs, that include T and B cells, dendritic cells, follicular dendritic cells, stromal cells, high endothelial venules and lymphatic vessels [63]. The interplay between elements from lymphatic system allows fluid homeostasis, adsorption of gastrointestinal lipids, immune response (traffic of antigen-presenting cells and lymphocytes) and metabolic balance [64, 65], thus

### Table 6.4 (continued)

| Model                     | Plastic type                  | Concentration | Time | Result                                                                                                                                                                                                 | References |
|---------------------------|-------------------------------|---------------|------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Mesenchymal stromal cells | Environmental microplastics   | <1 μm and <2.6 μm | N/A  | ↓ proliferating cells (around 30%) Alteration of differentiation potential. AMSCs remained in an early stage of adipocyte differentiation ↓ Bone Gamma-Carboxy glutamate Protein ↓ Osteopontin (SPP1) | [67]       |
| Stem cell fate            | Polystyrene nanoplastics      | N/A           | N/A  | ↑ GPX3 expression and ↑ HSP70 (ROS-related gene) and XBP1 (endoplasmic reticulum stress-related gene) expression ↑ MFN2 (mitochondrial fusion related gene) expression and ↑ FIS1 (mitochondrial fission related gene) ↑ proportion of cells in the S phase | [68]       |
| C57BL/6 J mice            | Polystyrene micro- nanoplastics | PS-MNPLs, 10 μm, 5 μm and 80 nm | 42 days | ↑ hematopoietic toxicity ↓ colony-forming, self-renewal and differentiation capacity ↑ proportion of lymphocytes ↑ hematopoietic injury | [69]       |
| C57BL/6 mice              | polystyrene microplastics     | (0.1 mg and 0.5 mg) of 5 μm | 28 days | ↑ white blood cell count ↑ increased Pict count ↓ growth of colony-forming unit CFU-G, CFU-M and CFU-GM | [70]       |
| Murine splenic lymphocytes| Nanoplastic                   | N/A           | N/A  | ↓ cell viability ↓ cell apoptosis ↓ apoptosis-related protein expression Altered mitochondrial membrane potential, and impaired mitochondrial function ↑ oxidative stress and mitochondrial structural damage ↑ induced endogenous apoptosis ↑ expression of activated T cell markers on the T cell surface ↑ differentiation of CD8 + T cells and the expression of helper T cell cytokines | [71]       |

*N/A: Not available*
the effect of microplastics has taken attention in view of the relevant role played for this complex system in pathophysiological processes [66].

When exposed to water bottle-derived MP, bone marrow mesenchymal stromal cells and adipose mesenchymal stromal cells showed defective differentiation pathways and decreased cell survival, which was related with increased senescence or apoptosis [67]. Surprisingly, the presence of modified-NP (amine free and decreased crosslink density) promoted ROS elimination in human bone marrow-derived mesenchymal stem cells via 70 kilodalton heat shock protein (HSP70) and X-box binding protein 1 (XBP1) downregulation (ROS- and endoplasmic reticulum stress-related genes, respectively), improved mitochondrial fusion, increased cellular proliferation, and adipogenic differentiation. However, long-term exposure showed increased cytotoxicity [68].

Intragastric administration of MP and NP to mice for 42 days promotes hematological toxicity as indicated by cellular changes in bone marrow, decreased colony-formation capacity, proliferation and differentiation, and increased lymphocyte numbers. Splenic abnormalities were also identified, especially a decrease in spleen weight, increased ultrastructural modifications, and the production of lipid droplets in mice exposed to MP and NP. In addition, plastic particles disrupted gut homeostasis by increasing dysbiosis, inflammation, and altered gut metabolism, which was linked to increased hematotoxicity [69]. Similarly, mice given MP orally for 28 days showed significant particle accumulation in the abdomen and limb bones, a decrease in white blood cell count, colony-forming ability, genomic and cell signal alterations related to T-cell homeostasis, osmotic stress, structural organization, and metabolism in bone marrow cells [70].

Finally, in Murine splenocytes, NP accumulation, especially of positive charged particles, was found, decreasing cell viability, upregulated apoptosis-related proteins, ROS production and mitochondrial dysfunction triggering impaired T cell activation, differentiation and cytokine expression [71].

6.6 Digestive System

The digestive system is composed of gastrointestinal tract (oral cavity, pharynx, esophagus, stomach, small intestine, and large intestine) and the accessory organs (teeth, tongue, and glandular organs such as salivary glands, liver, gallbladder, and pancreas) [72] mediating primary functions such as digestion, absorption, excretion and protection [73]. Interestingly, gastrointestinal alterations have been found to play an essential role in systemic inflammation, and even associated exacerbated poor outcomes as organ failure and death in view of the pathogenic interaction between digestive organs (from gastrointestinal tract and accessories) with muscles, lungs and even brain [74] (Table 6.5).

6.6.1 Gut

Food packages, drinking water and even fruits and vegetables have shown plastic traces representing a risk of dietary intake [75–77]. Regarding to this, experimental studies has shown that disposable plastic containers independent of frequency of use are associated to important gut microbiota alterations that were consistent with dysbiosis observed in mice treated with MP and NP. In fact, was demonstrate that the use of plastic containers also induces oral microbiota alterations that could not be prevented with reduction in the size and amount of microparticles consumed [78]. The oral exposure of mice to MPs for 6 weeks showed relevant histological alterations including increased intestinal crypt depth and defective recruitment of immune cells linked to increased alterations in intestinal microbiota [79]. Afterwards, intestinal histology of zebrafish exposed to MP and NP was analyzed and the findings showed that smaller plastic particles promoted worst intestinal epithelial damage and decrease gut microbiome diversity [80]. That was concordant with complementary studies showing that NP rather than MP, exacerbated gut dysbiosis and inflammation in adult zebrafish [81]. Remarkably, additional
Table 6.5 Gastrointestinal system-related effects of small plastic according to experimental model or sample, plastic type, experimental concentration used, experimental time and main findings

| Model                        | Plastic type               | Concentration                        | Time  | Result                                                                 | References |
|------------------------------|----------------------------|--------------------------------------|-------|------------------------------------------------------------------------|------------|
| Gut and oral microbiota      | Micro and nanoplastics     | 200 ug and 500 ug                    | N/A   | ↑ gastrointestinal dysfunction and cough. ↑ altered gut and oral microbiota ↓ gut bacteria | [78]       |
| Mice                         | Polyethylene microplastics | 100 µg/g of food                     | 6 weeks | ↑ Crypt depth throughout the intestinal tissues, defective recruitment of some intestinal immune cells was and intestinal dysbiosis | [79]       |
| Zebrafish                    | Micro and nano polystyrene plastics | 45–85 and 40–54 nm                  | 30 days | ↑ intestinal epithelial damage The gut microbial communities were affected by the combined exposure to microplastics | [80]       |
| Zebrafish                    | Micro and nanoplastics     | 10 µg/L and 1 mg/L of MPs (8 µm) and NPs (80 nm) | 21 days | ↑ microbiota dysbiosis in the gut of zebrafish ↑ flora diversity of gut microbiota ↑ Proteobacteria ↓ Fusobacteria, Firmicutes and Verrucomicrobiota. ↓ Actinobacteria decreased in the MPs treatment ↑ NPs treatment groups ↑ Aeromonas MPs and NPs treatment groups ↑ mRNA levels of il8, il10, il1β and tnfα in the gut | [81]       |
| Mice                         | Polystyrene micro and macroplastics | N/A                                  | 28 days | ↑ injuries to the gut tract, ↓ expression of tight junction proteins ↑ toxic effect of gut microbiota dysbiosis in M/NPLs-induced gut barrier dysfunction | [82]       |
| Mice                         | Polystyrene nanoparticles  | 3 and 223 µg/kg body weight          | N/A   | ↑ lipolysis under β-adrenergic stimulation in adipocytes in vitro and ex vivo ↑ lipid mobilization in obese mice and subsequently contributed to larger adipocyte size in the subcutaneous WAT ↑ macrophage infiltration in the small intestine ↑ lipid accumulation in the liver | [83]       |
| Mice                         | Polystyrene micro and nanoparticles | 50 nm PS, PS50; 500 nm PS, PS500; 5000 nm PS, PS5000 | 24 h   | ↑ permeability in the mouse intestines ↑ severe dysfunction of the intestinal barrier ↑ reactive oxygen species (ROS)-mediated epithelial cell apoptosis in the mice | [84]       |
| Human intestinal organoids   | Polystyrene nanoplastics   | 10 and 100 µg/mL                     | N/A   | ↑ cell apoptosis and inflammatory response ↑ endocytosis in the PS-NPs uptaking into enterocyte cells | [85]       |
| Human Intestinal             | Polystyrene nanoparticles  | N/A                                  | N/A   | Changes at the ultrastructural and molecular levels                    | [86]       |

(continued)
| Model                             | Plastic type                  | Concentration       | Time      | Result                                                                 | References |
|----------------------------------|-------------------------------|---------------------|-----------|------------------------------------------------------------------------|------------|
| Caco-2 Cells                     |                               |                     |           | This would indicate that no DNA damage or oxidative stress is observed  |            |
|                                  |                               |                     |           | in the human intestinal Caco-2 cells after long-term exposure to PSNPs |            |
| Human Intestinal Caco-2 Cells    | Polystyrene Nanoplastics      | 0, 10 and 100ug/ml | 24 h      | ↑cytotoxic effects modulate the cell's uptake of silver and slightly     | [87]       |
|                                  |                               |                     |           | modify some harmful cellular effects of silver, such as the ability to   |            |
|                                  |                               |                     |           | induce genotoxic and oxidative DNA damage                             |            |
| Small intestinal epithelium      | Micro and nanoplastics        | N/A                 | N/A       | ↓viability ↑permeability to 3 kD dextran                               | [88]       |
|                                  |                               |                     |           | ↑diffusion.                                                             |            |
|                                  |                               |                     |           | ↑phagocytosis                                                          |            |
|                                  |                               |                     |           | ↑endocytosis                                                           |            |
| Normal human colon cells         | Polystyrene micro and         | N/A                 | N/A       | ↑metabolic changes under both acute and chronic exposure by inducing    | [89]       |
|                                  | nanoplastics                  |                     |           | oxidative stress,                                                     |            |
|                                  |                               |                     |           | ↑glycolysis via lactate to sustain energy metabolism and glutamine      |            |
|                                  |                               |                     |           | metabolism to sustain anabolic processes                               |            |
| Human intestinal barrier         | Polystyrene nanoplastics      | 24 h                |           | ↑ MNPLs uptaken                                                         | [90]       |
| Mice                             | Polystyrene nanoplastics      | N/A                 | N/A       | ↑pathological changes in the liver,                                    | [91]       |
|                                  |                               |                     |           | ↑excessive autophagy through altered expression levels of PI3K, mTOR,   |            |
|                                  |                               |                     |           | Beclin-1, ATG5, LC3 and P62                                             |            |
|                                  |                               |                     |           | ↑apoptosis in the liver, ↑pyroptosis in the liver through NLRP3/Caspase-1|            |
|                                  |                               |                     |           | pathway via targeting NLRP3, ASC, Pro-Caspase-1, GSDMD and Cleaved-      |            |
|                                  |                               |                     |           | Caspase-1 expressions                                                  |            |
|                                  |                               |                     |           | pathological changes in the liver,                                     |            |
|                                  |                               |                     |           | ↑visceral organ injury, hepatotoxicity and impaired lipid metabolism    | [92]       |
| Mice and HL7702 cells            | Polystyrene Microplastics     | 0.1 and 1 mg/L      | 60 days   | Aggregations of neutrophils and apoptotic macrophages in the abdomen   | [93]       |
|                                  |                               |                     |           | of the larvae, ↑hepatic inflammation in the larvae, ↑fabbp10a in the   |            |
|                                  |                               |                     |           | larval livers                                                           |            |
| Zebrafish                        | Polystyrene nanoplastics      | 0.1 mg/ml           | N/A       | ↑aggregations of neutrophils and apoptotic macrophages in the abdomen   | [94]       |
|                                  |                               |                     |           | of the larvae, ↑hepatic inflammation in the larvae, ↑fabbp10a in the   |            |
|                                  |                               |                     |           | larval livers                                                           |            |
|                                  |                               |                     |           | ↑metabolic pathways of catabolic processes, amino acids, and purines    |            |
|                                  |                               |                     |           | ↑steroid hormone biosynthesis in zebrafish larvae, which may lead to    |            |
|                                  |                               |                     |           | the occurrence of immune-related diseases                               |            |
findings demonstrated that plastic-induced gut barrier disruption is mediated by the enhanced gut microbiota dysbiosis [82].

Experiments in mice exposed to NP in drinking water showed accumulation of NP in white adipose tissue and stomach viscerally and subcutaneously, associated to increased macrophage infiltration to small intestine. Then in vitro exposition of preadipocytes and adipocytes to NP at similar concentrations as reported in clinical studies (about $10^{10}$ particles/ml of 60 nm-sized) promoting a major internalization in preadipocytes within 4-8 h and reduced lipolysis under β-adrenergic stimulation [83]. Moreover, mice co-exposed to MP and NP showed increased intestinal permeability and toxicity induced by increased ROS production and epithelial cell apoptosis, being prevented with antioxidant pre-treatment [84].

Studies in intestinal organoids exposed to NP showed that plastic particles are accumulated in intestinal cells promoting inflammatory response and apoptosis. Interestingly, the use of inhibitors of clathrin-mediated endocytosis ameliorate the uptake of plastic particles by enterocytic cells [85]. Moreover, subtoxic polystyrene NPs exposure for 8 weeks, showed time-dependent internalization and ultrastructural changes in undifferentiated Caco-2 cells [86]. Similarly, Caco2 cells exposed to NPs showed exacerbated uptake and internalization was even observed in the nucleus, interestingly, at used concentrations these particles do no promoted exacerbated cytotoxic response, genotoxic response or oxidative damage [87].

In an in vitro model of intestinal epithelium, the use of environmentally relevant MP and NP increased permeability and reduced viability. Interestingly, cells exhibited mixed internalization mechanisms suggesting diffusion and phagocytosis of plastic particles [88]. Normal human intestinal CCD-18Co cells as model of healthy colon cells exposed to MP and NP at different concentrations for 48 h or 28 days showed interesting results. At 48 h, NP are highly internalized by colon cells, reaching up to 90% instead the 20% showed by MP. Despite de high mobilization into colon cells, NP and MP do not promote cytotoxicity or mortality, but the lowest concentrations of NP increased ROS production. Posteriorly, cancerogenic potential was evaluated and metabolic results showed glutamate and glutathione metabolism alterations strongly associated to metabolic rewiring. At long-term

| Model          | Plastic type                  | Concentration | Time   | Result                                                                 | References |
|---------------|-------------------------------|----------------|--------|------------------------------------------------------------------------|------------|
| Liver HepG2 cells | Polystyrene micro and nanoplastics | 0.1–100 μg/mL  | 1–24 h | ↑ toxicity ↑ intracellular concentrations ↑ apoptotic cell death ↑ upregulated interleukin-8 | [95] |
| Caco-2 and HepG2 Cells | Nanoplastics                      |                |        | ↑ DNA strand breaks pair ↑ reactive oxygen species production or changes in cell cycle distribution | [96] |
| Rats          | Nylon microplastic and nanoplastics | N/A            | N/A    | rats excrete smaller MPs more slowly than the bigger ones PA66-NP can pass through the gut barrier and entered the blood circulation | [97] |
| Mice          | Polystyrenes nanoplastics       | N/A            | N/A    | Long-term accumulation and in vivo inert property of nanoplastics ↑ health risks | [98] |

*N/A: Not available*
exposure, a 90% of MP was internalized, ROS production was increased in both MP and NP with partial recovery in cells with intermittent exposure, although metabolic function does not recover [89]. Finally, an in vitro model (differentiated Caco-2/HT29 intestinal cells and Caco-2/HT29 + Raji-B cells) of human barrier exposed to NP for 24 despite the uptake and dose-dependent translocation no significant genotoxic or oxidative damage was detected [90].

### 6.6.2 Liver

The effect of plastic particles on hepatic function has also been evaluated, showing for instance that arsenic- or polystyrene-derived NP promoted hepatotoxicity through increased ultrastructural markers of autophagy, apoptosis and pyroptosis, promoting liver injury in mice [91]. Moreover, oral administration of NPs increased organ injury, particularly in the liver, where altered lipid metabolism in mice through ROS/Pi3K/Akt signaling pathway [92]. Similarly, MPs from circulation promoted toxicity in hepatocytes through nuclear and mitochondrial DNA damage and enhanced proinflammatory cytokines expression by the activation of cGAS/STING pathway, ultimately promoting, liver injury and fibrosis even in low doses [93].

In zebrafish larvae, exposition to NP increased intestinal accumulation, abdominal neutrophil aggregation and macrophage apoptosis, liver-type inflammatory binding protein, ROS generation and metabolite alterations promoting hepatic inflammation [94].

Liver HepG2 cells showed high internalization of MP and NP, especially aminated-NP, which reduced cell viability. However, MP showed increased trend to promote cell death and inflammatory response, evidenced in elevated apoptosis and upregulation of IL-8 time-dependently [95]. Complementarily, HepG2 and Caco-2 cells exposed to NP from real-life food containers presented DNA break, showing the genotoxic effect of these particles [96].

### 6.6.3 Excretion

The dynamic of MP/NP uptake and clearance remains poorly documented in mammals, however, Peng and colleagues [97] feed rats with fiber and granular MPs and NPs for 7. The results showed that close to 90% and 94% of fiber and granular MP respectively, was eliminated in the feces, and interestingly, this occurs within the first 48 h after ingesting. Conversely, NPs excretion just reached 70%, being the major proportion (54%) also excreted within the first 48 h after intake. The reduced excretion rate showed for these particles suggested absorption by digestive tract which was further confirmed with the presence of NPs in rat plasm, indicating that ingested NPs cross gut barrier, enter to the circulation, accumulate in other organs as liver, spleen and lung and keep structural stability for at least 28 days [98].

### 6.7 Reproductive System

In humans, the presence of plastic particles (Table 6.6) has been reported in placenta and meconium from newborns [99]. Similarly, placenta from 6 donors presented of 12 types of plastic particles with distinct size, chemical composition and pigmentation in diverse placental portions, including maternal, fetal and amniochorial membranes [100]. Further, the same group reported the presence of microplastics in human placenta that correlated with unusual alterations in organelles from placenta, in mitochondria and endoplasmic reticule. In this work, the authors also informed plastic-consuming habits of pregnant women, showing a high prevalence of plastic wrapped food, drinking in plastic containers, use of cosmetics with synthetic polymers, including shower gel, creams, soap, toothpaste and chewing-gum consume. The localization of plastic particles included inner and outer membrane of villus layer, lysosomes, peroxisomes, vacuoles and lipid droplets intracellularly, stroma, pericytes and endothelial cells in extracellular space [101].
Table 6.6 Reproductive system-related effects of small plastic according to experimental model or sample, plastic type, experimental concentration used, experimental time and main findings

| Model                          | Plastic type          | Concentration | Time   | Result                                                                                                                                                                                                 | References |
|--------------------------------|-----------------------|---------------|--------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Human placenta and fetal meconium | Micropalstics         | N/A           | N/A    | Human placenta and meconium samples were screened positive for polyethylene, polypropylene, polystyrene, and polyurethane, of which only the latter one was also detected as airborne fallout in the operating room—thus representing potential contamination | [99]       |
| Human placenta                 | Microplastics         | N/A           | N/A    | Accumulation in fetal side, maternal side and chorioamniotic membrane of human placenta                                                                                                                   | [100]      |
| Human placentas                | Microplastics         | N/A           | N/A    | oxidative stress, apoptosis, and inflammation, characteristic of metabolic disorders underlying obesity, diabetes, and metabolic syndrome and partially accounting for the recent epidemic of non-communicable diseases | [101]      |
| Mice                           | Polystyrene nanoplastic | N/A           | N/A    | Suppressive effects on embryo development
  ↑ increased frequency of congenital abnormalities (especially in the nervous system), including neural tube defects
  ↑ autophagy. ↑apoptotic cell death                                                                                                                   | [102]      |
| Zebrafish                      | Polystyrene nanoplastics | 10 mg/L       | 120 days | Bioaccumulation of TDCIPP
  ↑ parental transfer of TDCIPP to their offspring
  ↑thyroid disruption in adults, and then leads to thyroid endocrine disruption in their larval offspring.
  ↑transgenerational thyroid endocrine disruption,
  ↑T4
  ↑ thyroglobulin (tg), uridine diphosphate-glucuronosyltransferase (ugt1ab), thyroid-stimulating hormone (tshβ), and thyroid hormone receptor (trα) expression in the F1 larvae | [103]      |
| Human placental cells          | Polystyrene nanoparticles | N/A           | N/A    | Toxicity induced on human placental cells. ↑ cytotoxicity, inhibition of protein kinase A (PKA) activity, oxidative stress, and cell cycle arrest
  ↑intracellular reactive oxygen species in human placental cells, ↑DNA damage and lead to cell cycle arrest in G1 or G2 phase, inflammation and apoptosis | [104]      |
| Model                     | Plastic type     | Concentration | Time         | Result                                                                                                | References  |
|---------------------------|------------------|---------------|--------------|-------------------------------------------------------------------------------------------------------|-------------|
| Human trophoblast         | Polystyrene      | 100 ug/ml     | 24 h         | [cell viability caused cell cycle arrest cell migration and invasion abilities level of intracellular reactive oxygen species and the production of proinflammatory cytokines (TNF-α and IFN-γ)] | [105]       |
| Mice                      | Polystyrene      | 1 and 10 mg/L | N/A          | [fetal weights Abnormal morphologies of cells in the placenta and fetus Disturbed cholesterol metabolism and complement and coagulation cascades pathways] | [106]       |
| Human placenta cells      | Micro and        | N/A           | N/A          | [number of unique features in pristine particles DAMAGE to the plasma membrane hsd17b1]               | [107]       |
| Mouse Embryonic           | Polystyrene      | 200 mg/L      | 2 days       | [Plastic accumulation at the juxtanuclear position]                                                | [108]       |
| Fibroblasts               | Nanoplastics     |               |              |                                                                                        |             |
| Mouse Embryonic           | Polystyrene      | N/A           | N/A          | [Oxidative and inflammatory stress caused Affect cellular functions]                            | [109]       |
| Fibroblasts               | Nanoplastics     |               |              |                                                                                        |             |
| Mouse                     | Polystyrene      | 0.1, 1 and    | From pregnancy until postnatal day 21 | [Birth and postnatal body weight in offspring mice liver weight oxidative stress, caused inflammatory cell infiltration, proinflammatory cytokine expression Disturbed glycometabolism disrupted seminiferous epithelium and decreased sperm count in mouse offspring testicular oxidative injury malondialdehyde generation and altered superoxide dismutase and catalase activities infiltration, proinflammatory cytokine expression] | [110]       |
| Neonatal rat ventricular | Polystyrene      | N/A           | N/A          | [Mitochondrial membrane potentials and cellular metabolism]                                       | [111]       |
| myocytes                  | Nanoplastics     |               |              |                                                                                        |             |
| Cultured neural stem      | Polystyrene      | N/A           | N/A          | Altered the functioning of NSCs, neural cell compositions, and brain histology in progeny Molecular and functional defects were also observed in cultured NSCs in vitro abnormal brain development neuropathological and cognitive deficits | [112]       |
| cells (NSCs) and mice     | Nanoplastics     |               |              |                                                                                        |             |
| Mice                      | Polystyrene      | 1 and 10 mg/kg| 35 days      | [Acrosome integrity and acrosome reaction, altered testicular ultrastructure]                     | [113]       |

*N/A: Not available*
These findings further were confirmed in mice, where NPs crossed the placenta and affected offspring development. In addition, studies about embryo/fetal development showed increased congenital abnormalities, especially impaired neural tube formation mediated by enhanced endocytosis, autophagy activation (unrelated with plastic degradation) and further cytoplasmic accumulation promoting apoptosis [102]. Additionally, in zebrafish exposed to MP and NP for 120 days was observed parental intestinal, branchial, sexual organ and hepatic accumulation, being enhanced in female animals, and decreased T4 thyroid hormone production. Interestingly, transference of plastic particles was observed in the offspring eggs and transgenerational thyroid disbalance [103].

Mechanisms involved in maternal and fetal interactions with plastic particles has described the adverse effect of amino-charged NP, considering that after exposure downregulated protein kinase A (PKA) activity, increase oxidative stress and cell cycle arrest in human placental cells promoting inflammation and apoptosis [104]. Similarly, experiments performed in human trophoblast that NP were captured to cytoplasm, affecting cell viability, cell cycle arrest, cell migration and invasion, increased ROS and the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) [105].

Importantly, further evidence has shown that maternal exposure to NP reduced fetal growth that could be mediated by altered cholesterol transportation in fetal skeletal muscle and placenta. Additionally, transcriptional analysis performed demonstrated alteration in coagulation genes [106]. Complementary studies observed in placental cells that MP and NP do not affected viability, but NP was highly captured by cells and promoted membrane damage [107].

In mouse embryonic fibroblasts (MEF) exposed to NP was observed increased uptake of particles with juxtanuclear accumulation that was associated to retrograde transport along microtubules. Blocking histone deacetylase 6 resulted in rapid exocytic NP clearance and interestingly, the inhibition of retrograde transport of NP prevented pro-inflammatory and pro-oxidative status [108]. Similarly, MEF exposed to NPs also accumulated in cytoplasm after endocytic entry, with subsequent oxidative, inflammatory damage and impaired autophagy activation associated to enhanced accumulation in lysosomes or autolysosomes. Remarkably, accumulation of NPs was alleviated after prolonged cell culture, suggesting that plastic particles are removed from the cells, although associated mechanisms remain unclear [109].

The trans-generational effect of NP was explored in mice exposed to plastic particles during pregnancy and lactation periods and findings showed reduced body and liver weight in the offspring. Interestingly, male offspring presented important alterations including increased ROS, cell infiltration, inflammatory cytokines, impaired carbohydrates metabolism in liver and reduced testis weight, seminiferous epithelium integrity and sperm count associated to elevation in oxidative testicular injury [110]. Similarly, neonatal cardiomyocytes presented altered contractility after positively charged NP exposure [111] and maternal exposure to high NP dose in gestation and lactation induced impaired neural stem cell functioning, neuronal composition and cerebral histology in the offspring triggering neurophysiological and cognitive alterations, showing the harmful potential of plastics after crossing blood-milk barrier [112].

Finally, the reproductive toxicity induced by plastic particles was demonstrated in mouse spermatocytes where exposition to NP for 35 days decreased sperm quality and altered testicular structures, as evidenced the reduction of acrosome reaction, integrity, biogenesis and impaired testicular ultrastructure [113].

6.8 Predisposition to Diseases

In acute inflammatory settings, for instance endotoxemia, it was observed that polystyrene NP exacerbate the harmful response to lipopolysaccharide (LPS), promoting upregulated reactive oxygen species (ROS) production, worsened myocardial morphology linked to
increased expression fibrosis-associated proteins and increased autophagy via ROS/TGF-β1/Smad pathway [114]. Complementarily, RAW2647 cells stimulated with LPS, and NP presented enhanced oxidative stress through Mitogen-activated protein kinase (MAPK) pathway and endotoxemic mice injected with NP undergone worsened splenic inflammation and necroptosis via ROS/MAPK [115]. Likewise, endotoxemic mice exposed to NP worsened ROS production, oxidative stress through NF-κB/NLRP3 pathway, decreasing tight junction proteins and pro-inflammatory cytokines production resulting in exacerbated duodenal inflammation and permeability [116].

In diabetic mice, the exposure to MPs exacerbated hepatic damage, alterations in lipid metabolism, inflammatory response and intestinal dysbiosis [117]. In mice with normal diet and high-fat diet and then exposed to MP presented increased insulin resistance, plasma LPS, TNF-α and IL-1β and enhanced dysbiosis [118]. Further findings confirmed that MPs exposure promoted insulin resistance and even diabetes in mice [119].

Mice with previous intestinal immune imbalance and then exposed to MPs presented exacerbated response to MPs, evidenced in the enhanced accumulation of plastic particles and subsequent release of pro-inflammatory mediators such as TNF-α, IL-1β and IFN-γ, worsened histological damage in colonic mucosa, disturbance of microbiota and impaired metabolism, showing that the presence of immune disturbances increases susceptibility to plastic side effects [120].

In a mice model of non-alcoholic fatty liver disease (NAFLD), the intravenous administration of NP showed exacerbated hepatitis mediated by interference in lipid metabolism, increased infiltration of Kupffer cells and enhanced oxidative status associated to the reduction of SOD activity. In addition, NP exposure promoted excessive collagen production, aggravating fibrosis and liver injury [121].

Moreover, considering the vast evidence demonstrating the adverse effects of plastics in gastrointestinal system, the effect of MP was evaluated under pathological conditions in dextran sodium sulfate (DSS)-induced colitis. Results showed that in healthy mice, MP exposure promoted minimal effect on intestinal barrier integrity and liver function, whereas in animals with colitis, MP promoted colon shortening, histological damage, inflammation, reduced mucosa and increased colon permeability. Additionally, increased secondary liver injury risk [122].

The evaluation of cancerogenic effect of plastic particles exposure was carried in prone-to-transformed progress MEF cells exposed by 6 months. Importantly, the authors observed that cancer markers including the linked to premature tumoral phenotype (stress related genes or microRNA alteration), late tumoral phenotype (growing and migration ability) and tumoral aggressivity (invasion, pluripotentiality, tumorspheres formation) were intensified in cells exposed to plastic-derived particles [123]. Similarly, mouse embryonic fibroblast exposed to MP and NP for 12 weeks, presented aggravated DNA damage and acquisition of an oncogenic phenotype featured by morphological changes, aggressivity and invading potential [124].

6.9 Concluding Remarks

Taken together, clinical and experimental data highlighted the hazardous potential of plastic particles beyond their environmental influence, since has been detected in human samples, raising concerns about their unknown implications. The current analysis gives interesting mechanistic insights into the local and systemic action in practically all tissues, as well as its role in inflammatory responses. However, further research is needed to determine long-term consequences, the interaction with comorbidities, transgenerational impacts, and, of course, therapeutical potential steps to avoid its negative side effects.
Statements and Declarations

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Impact of a Community-Based Pelvic Floor Kinesic Rehabilitation Program on the Quality of Life of Chilean Adult Women with Urinary Incontinence

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Abstract

The pelvic floor forms the primary bottom tissue of the pelvic cavity. It comprises muscles that play a fundamental role in bowel and bladder emptying. Alterations of pelvic floor muscles will result in dysfunctions such as urinary incontinence (UI). Given the high prevalence of UI and its impact on the quality of life (QoL) in patients with pelvic floor muscle dysfunctions, it is necessary to implement public, community, and generalized programs focused on treating these dysfunctions. **Objective**: To determine the effect of a community rehabilitation program on QoL, UI severity, and pelvic floor muscle strength in patients with UI. **Patients and Method**: A descriptive prospective cohort study. Twenty subjects between 44 and 75 years old with a diagnosis of UI, participants of a community kinesic rehabilitation program on the pelvic floor in Maipú, Santiago, Chile, were evaluated. These volunteers were intervened for six months, and QoL was measured with the 36-Item Short-Form Health Survey (SF-36) and International Consultation on Incontinence Questionnaire Short-Form (ICIQ-SF) scales, UI severity with the Sandvick test, and pelvic floor muscle strength with the Oxford scale. Patients were followed up three months post-intervention. **Results**: Significant improvements were observed in all scales after applying for the community kinesic rehabilitation program, and the changes were maintained at a 3-month follow-up. **Conclusions**: Since the improvement in QoL, UI severity, and pelvic floor muscle strength after the intervention, it is relevant to consider the implementation of community programs aimed at education, screening, and early rehabilitation of these patients.

Keywords

Urinary incontinence · Pelvic floor disorders · Quality of life · Rehabilitation · Community health planning

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| BMI          | Body mass index |
| COVID-19     | Coronavirus disease 2019 |
| HR-QoL       | Health-Related Quality of Life |
| ICIQ-SF      | International Consultation on Incontinence Questionnaire Short-Form |
| ICS          | International Continence Society |
| KHQ          | King's health questionnaire |
| MMSE         | Mini-mental State Examination |
| PFM          | Pelvic floor muscles |
| QoL          | Quality of life |
| SF-36        | 36-Item Short-Form Health Survey |
| TENS         | Transcutaneous electrical nerve stimulation |
| UI           | Urinary incontinence |
| VAS          | Visual Analog Scale |

7.1 Introduction

Urinary continence results from the anatomical and functional integrity and coordination of the lower urinary tract, composed of the bladder and urethra. It is under the influence of the central, peripheral, and autonomic nervous systems that coordinate the filling and emptying phases of the bladder through inhibitory and facilitating actions on the smooth and striated musculature that form the functional unit of the urinary tract and where the pelvic floor plays a fundamental role [1, 2].

The pelvic floor forms the bottom of the pelvic cavity. It comprises a series of muscles, fasciae, nerve, and vascular structures enclosing the pelvis’s lower part [3, 4]. The pelvic cavity also contains the pelvic organs, such as the bladder, uterus, cervix, vagina, and rectum, which are directly related to the pelvic floor muscles (PFM) [5]. Among the functions of the PFM, mainly the levator ani and coccygeus, is to reduce the displacement of the bladder neck, stabilize the vaginal wall and actively participate in the micturition process by contracting and relaxing them in synergy with the muscles of the abdominal wall, detrusor, internal and external urethral sphincters [4, 6]. These muscles can be consciously activated to help stabilize the anterior vaginal wall and urethra [5, 6]. Besides, these muscles play a fundamental role in visceral support, bladder and digestive containment and emptying, sexual functions, and the movement of the fetus during delivery. Therefore, their alteration will result in pelvic floor dysfunction related to clinical alterations, including urinary incontinence [3, 5].

Urinary incontinence (UI) is the involuntary loss of urine or involuntary urination [7]. There are different types of UI. The International Continence Society (ICS) has classified UI [8] as (A) Stress, when urethral closure pressure is insufficient, or there is excess urethral mobility due to weakness of pelvic floor muscles that upon a sudden increase in abdominal pressure from activities such as running, coughing, sneezing or laughing, results in urine leakage [4, 8]. (B) Urgency, when there is increased contractility of the bladder, i.e., an excess of uncontrolled detrusor muscle activity during the filling phase [6, 8] and (C) mixed, the association of symptoms of involuntary urine leakage on exertion with symptoms of urgency [8–10].

UI is a health problem whose incidence varies between 30 and 60% [9]. UI-related dysfunctions impact quality of life (QoL), in issues such as social, psychological, physical, occupational, and sexual [9, 11]. The prevalence of UI in the female life cycle is directly related to age, the number of pregnancies, childbirths, and hormonal changes [11].

Given the high prevalence of UI and the impact on the QoL of people with pelvic floor muscle dysfunctions, it is necessary to implement programs focused on treating these dysfunctions. Under this perspective, we characterize clinical- and socio-demographically patients with pelvic floor dysfunctions from a hospital belonging to the public health system. Specifically, in the volunteers with a medical diagnosis of UI, we sought to determine the effect of applying for a
community rehabilitation program in the QoL, the severity of UI, and pelvic floor muscle strength.

7.2 Methods

Type of Study: Descriptive prospective cohort study. In August 2021, a community pelvic floor rehabilitation program called “Esencia de Mujer” (Essence of a woman) was developed and financed by 6% competitive funds from the National Fund for Regional Development (FNDR by its acronym in Spanish).

Participants: Participants were recruited through an open call aimed at women with pelvic floor dysfunctions through mass media on social networks and placement of advertising posters in physical spaces in the commune of Maipú in the Metropolitan Region, Santiago, Chile. To participate, they were asked to send their cell phone number. Attendance at an informative talk was arranged by telephone to explain the procedures involved in the study and intervention in detail.

Sample: The sample included 22 women between 44 and 75 years old who freely accepted to participate in the research from the open call. Women living at Maipú in Santiago, Chile, met the inclusion criteria.

Inclusion criteria: Participants fulfilled the following inclusion criteria: female sex, belonging to Maipú, age range between 44 and 75 years, with a confirmed medical diagnosis of UI regardless of its clinical type, and able to understand simple commands, according to Mini-mental State Examination Spanish version (MMSE) [12].

Exclusion criteria: Participants who presented any of the antecedents described in the following criteria were excluded from this study by self-report of the users or by the clinical interview: Mental or physical disability that limited the performance of the protocol, interdiction, having an acute pathology that directly or indirectly influenced pelvic floor dysfunction at the time of the evaluation, consumption of drugs that could interfere with the interventions, presence of cardiometabolic pathologies that generate confounding variables, pregnancy, urinary tract or skin infections, stage 3 and 4 prolapse. Thus, a total sample of 22 participating women was obtained.

The users who had a pelvic floor dysfunction diagnosis and were excluded from the study were not part of the reports of this research. Nevertheless, they did receive therapy corresponding to their condition.

7.3 Ethical Aspects:

This study was approved by the Scientific Ethics Committee of the Universidad de las Américas (Project ID: CEC_FP_2021009).

Informed Consent and Data collection: Before the incorporation of the users who wished to participate in the study, they were given information concerning the study during the first session, voluntarily determining their participation by signing an informed consent form. During the first session, the background information of the participating users was collected and encrypted to protect their identity.

Subsequently, the Spanish version of the MMSE questionnaire was applied by employing an individual interview [12].

Anthropometric measurements: Basic parameters were measured, such as weight, height, and calculation of body mass index (BMI), according to Keys et al. [13].

Kinesic evaluation: A global evaluation was performed, including posture, abdominal and diaphragmatic musculature. Then, an evaluation of pelvic floor muscle contraction by manual testing in the supine decubitus position, with flexion and abduction of the hips, flexion of the knees and feet supported, with the genital region and lower limbs bare [14, 15]. The physical examination consisted of visual inspection, palpation, and muscle assessment [15, 16].

To perform vaginal touch and to manually assess the perineal musculature, the index and middle fingers were introduced into the vaginal introitus previously lubricated, following the direction of the vaginal canal obliquely downward and backward. Then, the pelvic floor
The muscle contraction assessment was measured according to the protocol published by Laycock, with its acronym PERFECT (P = power, E = endurance, R = repetitions, F = fast, ECT = every contraction timed) [16, 18]. The user was asked to maintain the contraction strength until a 50% loss of strength was detected, with a maximum of 10 s [17, 19]. Muscle fatigue was assessed by the number of contractions with a maximum of 10 that the patient could perform with maximum resistance recorded, with a rest time of 4 s between contractions [15, 18]. For contraction speed, we asked the user to perform after a rest period of 1 min, the most significant number of rapid one-second contractions until muscle fatigue, with a maximum of 10 [15, 16].

The type of incontinence was classified according to the characteristics of incontinence episodes following the guidelines of the International Continence Society (ICS) [8].

**Perineal muscle strength using the modified Oxford scale**: The contractile capacity of the pelvic floor muscles was quantified by digital assessment [15, 20]. According to this scale, strength was categorized with a value between 0 and 5. This evaluation was performed pre- and post-intervention.

**Quality of life (QoL)**: Two self-administered questionnaires in Spanish were used: the 36-Item Short-Form Health Survey (SF-36) v2 [21] and the International Consultation on Incontinence Questionnaire Short-Form (ICIQ-SF) [22], which sought to evaluate QoL globally and specifically concerning UI, respectively.

**Severity of Urinary Incontinence (UI)**: The Sandvick severity test [23] was used to evaluate the symptomatology severity of the participants with UI, asking 2 questions to categorize its degree into mild, moderate, severe, and very severe.

The QoL and UI severity questionnaires were applied pre-intervention, immediately post-intervention, and 3 months post-intervention.

**Pain assessment**: In patients with pain during the evaluation, the Visual Analog Scale (VAS) [24] was applied to quantify pain in the pelviperineal area. If the pain was limiting for the session, priority was given to treating the pain and then receiving therapy corresponding to IU. In patients whose pain was not limiting for the kinesic session, pain and UI were treated together.

**Kinesic treatment**: This consisted of the following interventions:

**Education regarding pelvic floor dysfunction**: This was accomplished through presentations that included images and delivery of educational material regarding her pathology and the role of the pelvic floor musculature in their recovery.

**Manual biofeedback**: Controls were performed in individual sessions implementing a program of specific muscle re-education exercises according to the Laycock muscle contraction assessment protocol [15, 16]. The therapist performed this protocol by inserting the index and middle fingers into the patient’s vaginal canal. Afterward, the patient was asked to perform a muscular contraction of the pelvic floor to evaluate her muscle strength. At the same time, a more remarkable contraction power or duration could be requested. Controls were carried out every 2 weeks, and each lasted 20 min.

**Specific and personalized pelvic floor exercise**: The home exercises for each case were dosed according to the progress evaluated in the controls using the PERFECT protocol [15, 16, 18]. According to the results obtained in the controls, exercise progressions were indicated. The participants performed the exercise regimen 2 times a day, 3 to 4 times a week.

**Functional training**: Finally, the volunteers who strengthened the perineal musculature with the home exercises and individual controls were referred to group sessions where functional training was practiced integrating the pelvic floor musculature, which consisted of a warm-up, followed by functional exercises of the upper limbs, trunk, and lower limbs, and finally stretching. For all the exercises, the focus was on breathing with an abdominal pattern and with
effort in the expiratory stage, activating the pelvic floor [25, 26]. An aerobic dance was performed during the warm-up for 7 min. The functional exercises of the upper extremities were performed with elastic bands: triceps, horizontal extension, arm flexion, and vertical push-up on the wall, all varying according to repetitions and resistance of the elastic band [25, 26]. Trunk exercises were performed on the floor: bird dog and bridge, all with body weight and progressions with elastic bands. Lower extremity exercises included squats and lunges with progressions [25, 26]. The intensity for all the exercises was moderate and measured by the Borg CR10 effort perception scale [27]. Based on this, during the first week, the participants performed 1 series of each exercise, reaching 2 series in the second week. The sessions had an extension of 50 min twice a week.

Neuromodulation of the tibialis posterior: Consisted of transcutaneous electrostimulation with Transcutaneous electrical nerve stimulation (TENS) current with a pulse amplitude of 200 ms, frequency 20 Hz, with regulated intensity until dorsiflexion of the greater brace and/or plantar flexion of the second to the fifth brace of 1 to 10 mA or according to the tolerance of the patient. An electrode was located 3 cm proximal and 1 cm posterior to the medial malleolus of the right or left leg, and another electrode was on the calcaneus [28–31]. It was applied 3 times per week.

Extension of the rehabilitation program: The total duration of participation in the program was 6 months.

Statistical analysis: Qualitative and categorical data were presented as relative percentages. Data analysis was performed using Graphpad Prism® 9 statistical software. Quantitative variables were evaluated for normality with the Shapiro–Wilk test. Variables that showed parametric behavior are reported with their respective mean and standard deviation. Variables that did not present normal behavior are reported with median and interquartile ranges. For the association between quantitative variables, the Wilcoxon rank test and the Friedman test were used as appropriate. Statistical significance was established with an alpha error of less than 0.05.

7.4 Results

Considering the 22 users evaluated, the mean age was $59.5 \pm 8.71$ years, the median weight was $69.45$ (59.83–83.05) kilograms, and the average height was $1.59 \pm 0.06$ m, with a median BMI corresponding to 27.22 (23.81–35.47). They received pelvic floor kinesic rehabilitation to treat their UI between August 01, 2021 and January 31, 2022 with a mean attendance to scheduled sessions of $80.35 \pm 22.95\%$.

Regarding the schooling of the sample, 81.36% had secondary or higher education, and 18.64% did not complete elementary education. About the number of pregnancies in the sample, 95.45% had at least one pregnancy, 4.55% had never been pregnant, 63.64% had undergone episiotomy at some time in their deliveries, and 86.36% did not have instrumentalized deliveries. Details of the demographic and anthropometric characteristics of the participants are shown in Table 7.1.

When the classification of the type of incontinence, according to the ICS described in Table 7.2, was analyzed, we found that in the initial evaluation, most of the sample presented a categorization of stress UI with 54.55%, followed by mixed UI with 31.82% and urgency UI with 13.64%. It should be noted that no patient was categorized as continent. However, the sample, 40.91%, was classified as continent at the end of the intervention, followed by emergency UI with 31.82%, stress UI with 22.73%, and mixed UI with 4.55%. At the 3-month post-intervention follow-up, 31.82% of the sample remained continent, 36.36% had urgency UI, 27.27% had stress UI, and 4.55% had mixed UI.

The assessments of UI severity can be seen in Tables 7.3 and 7.4.

Our study determined a significant improvement (p-value < 0.0001) in the ICIQ SF. The sample shows an initial median score of 10 (7.75–13–5) at the end of treatment of 5 (0–5).
### Table 7.1 Baseline characteristics of the sample

| Characteristic                  | Value          |
|--------------------------------|----------------|
| Total participants            | 22             |
| Age (years) [Mean ± SD]       | 59.5 ± 8.71    |
| Weight (kg) [Median (Q1–Q3)]  | 69.45 (59.83–83.05) |
| Height (m) [Mean ± SD]        | 1.59 ± 0.06    |
| BIM (Kg/m²) [Median (Q1–Q3)] | 27.22 (23.81–35.47) |

**Education (% of total)**

| Education                              | % of total |
|----------------------------------------|------------|
| Incomplete elementary school           | 4.55       |
| Complete elementary school             | 13.64      |
| Complete high school                   | 63.64      |
| Technical and vocational education     | 18.18      |

**N° of births (% of total)**

| N° of births | % of total |
|--------------|------------|
| 0            | 4.55       |
| 1            | 18.18      |
| 2            | 13.64      |
| 3            | 36.36      |
| 4            | 13.64      |
| 5            | 13.64      |

**Episiotomy (% of total)**

| Episiotomy | % of total |
|------------|------------|
| Yes        | 63.64      |
| No         | 36.36      |

**Instrumentalization in childbirth (% of total)**

| Instrumentalization | % of total |
|---------------------|------------|
| Yes (Forceps)       | 13.64      |
| No                  | 86.36      |

**Percentage of attendance to scheduled sessions [Mean ± SD]**

| Evaluation (%) of total | Mean ± SD |
|-------------------------|-----------|
| 80.35 ± 22.95           |

The variables age and attendance percentage are presented with their respective means and SD (standard deviation). The variables weight and body mass index (BMI) are presented with their respective median and quartile 1–quartile 3 (Q1–Q3). The variables education, number of births, episiotomy, and instrumentalization in childbirth are expressed as relative percentages of the total.

### Table 7.2 Classification of the type of incontinence according to the International Continence Society

| Type of incontinence | Evaluation (% of total) |
|----------------------|-------------------------|
|                      | Initial | Final | Follow-up |
| Continent            | 0       | 40.91 | 31.82     |
| Urgency              | 13.64   | 31.82 | 36.36     |
| Stress               | 54.55   | 22.73 | 27.27     |
| Mixed                | 31.82   | 4.55  | 4.55      |

The type of incontinence was categorized pre-intervention, post-intervention, and at a 3-month post-intervention follow-up. The categorizations are expressed in relative percentages of the total.
and a follow-up of 5 (0–6.25). In the specific quality of life question, which was “To what extent have these urine leaks, that you have, affected your daily life?” scoring from 1, which is nothing, to 10, the maximum, the sample shows as the initial result a median of 4.5 (3–6.25), at the end of treatment 2 (1–2) and follow-up 2 (1–3), obtaining positive and significant results (p-value < 0.0001) for our intervention. In addition, the results at the end of treatment were maintained 3 months post-intervention.

Pelvic floor muscle strength, evaluated with the Oxford scale, increased significantly (p-value < 0.0001). Our sample shows a median of 2 (1–3) as the initial result and at the end of treatment 3 (3–4).

In the Sandvik severity test, our study obtained a decreased severity of urinary incontinence (p-value < 0.0001), our sample gave an initial result of a median of 6 (3.75–6.5), at the end of treatment 1 (0–2), and maintained at follow-up 1.5 (0–2.25). In addition, we could observe that initially, 13.64% of the sample presented mild incontinence, 63.64% moderate, 13.64% severe, and 9.09% very severe. At the end of the study, 40.91% were continent, 50% presented mild incontinence, 9.09% moderate, 0% severe, and 0% very severe. At follow-up, 31.82% were continent, 45.45% showed mild incontinence, 18.18% moderate, 4.55% severe, and 0% very severe. Thus, we can observe that three months after the intervention, there was a high percentage of decreased severity even if there were continent patients.

It can be seen that all the evaluation scales showed significant changes toward the end of the intervention. It should be noted that the Oxford scale was applied only at the end of the program, and the Sandvik test and ICIQ-SF were applied at the 3-month follow-up.

Finally, the evaluation of the quality of life according to the SF-36 scale is detailed in Table 7.5.

| Table 7.3 | Assessment of severity of urinary incontinence |
| --- | --- |
| Test | Evaluation [Median (Q1–Q3)] | Initial | Final | Follow-up | p-value |
| Sandvick | 6 (3.75–6.5) | 1 (0–2) | 1.5 (0–2.25) | <0.0001 |
| ICIQ-SF | 10 (7.75–13.5) | 5 (0–5) | 5 (0–6.25) | <0.0001 |
| HR-QoL. ICIQ-SF | 4.5 (3–6.25) | 2 (1–2) | 2 (1–3) | <0.0001 |
| Oxford | 2 (1–3) | 3 (3–4) | – | <0.0001 |

Assessments of the Sandvick Test and ICIQ-SF were performed pre-intervention, post-intervention, and at a 3-month post-intervention follow-up. The Oxford scale was only applied pre and post-intervention. Each result has its respective median and quartile 1–quartile 3 (Q1–Q3). ICIQ-SF: International Consultation on Incontinence Questionnaire Short-Form; HR-QoL: Health-Related Quality of Life

| Table 7.4 | Classification of the severity of incontinence according to the Sandvick test |
| --- | --- |
| Category | Evaluation (% of total) | Initial | Final | Follow-up |
| Continent | 0 | 40.91 | 31.82 |
| Slight | 13.64 | 50.00 | 45.45 |
| Moderate | 63.64 | 9.09 | 18.18 |
| Severe | 13.64 | 0 | 4.55 |
| Very severe | 9.09 | 0 | 0 |

The type of urinary incontinence severity was categorized pre-intervention, post-intervention, and at a 3-month post-intervention follow-up. The Sandvick severity test categorizations are expressed as relative percentages of the total
In the SF-36 questionnaire, we observed significant improvements (p-value = 0.0007) in physical functioning at the end of the intervention with a median of 95 (83.75–100) points. At program follow-up, it even tended to increase with a median of 97.5 (82.5–100) points.

Regarding physical health limitations, these remained at a median of 100 from the initial evaluation to the 3-month follow-up (p-value = 0.0205).

In the limitations in emotional problems, there were no significant changes (p-value = 0.8395) with an initial median 100 (24.98–100) points. At the end of the intervention, there were improvements with a median of 100 (50.03–100) points, and at the follow-up stage, they tended to decrease with a median of 83.35 (0–100) points.

There were significant improvements in energy/fatigue (p-value = 0.0004). At the end of the treatment, a median of 72.5 (65–80) points was observed, while at the follow-up of the program, there was a decrease in the evaluated results with a median of 70 (50–75) points. These values remained higher compared to the median of 70 (53.75–76.25) points at the beginning of the intervention.

In the emotional role, we obtained significant improvements at the end of the intervention with a median of 72 (60–85) points. At the follow-up of the program, there was a tendency to decrease with a median of 62 (48–76) points (p-value < 0.0001).

In the social role, the tendencies were maintained at the end of the intervention with a median of 85.5 (71.88–100) points compared to the beginning with a median of 87.5 (71.88–100) points. At the follow-up of the program, it decreased by a median of 75 (59.38–100) points. These changes were not significant (p-value = 0.0891).

Regarding pain, our sample obtained significant improvements (p-value = 0.0013). At the end of the treatment, a median of 78.75 (67.5–90) points was observed, and at the follow-up of the program, there was a decrease with a median of 70 (43.13–90) points. This value was higher than at the beginning of the intervention, with a median of 70 (24.38–80) points.

There were significant improvements in general health (p-value < 0.0001). At the end of the intervention, there was a median of 75 (61.25–81.25) points. This value was higher than at the beginning of the intervention, with a median of 55 (47.5–70) points.

### 7.5 Discussion

There is vast evidence that UI is an underreported, underdiagnosed, and undertreated disease that diminishes QoL. Pelvic floor rehabilitation has a positive impact on the QoL of women with UI [10, 18, 28–34]. It has been seen

| Table 7.5 Evaluation of quality of life according to 36-Item Short-Form Health Survey |
|---------------------------------|----------------|----------------|----------------|----------|
| Sections                        | Evaluation SF-36 [Median (Q1–Q3)] | Initial | Final | Follow-up | p-value |
| Physical functioning            | 90 (68.75–96.25) | 95 (83.75–100) | 97.5 (82.5–100) | 0.0007   |
| Limitations in physical health  | 100 (0–100)      | 100 (93.75–100) | 100 (43.75–100) | 0.0205   |
| Limitations on emotional problems | 100 (24.98–100) | 100 (50.03–100) | 83.35 (0–100)  | 0.8395   |
| Energy/fatigue                  | 70 (53.75–76.25) | 72.5 (65–80) | 70 (50–75) | 0.0004   |
| Emotional role                  | 64 (51–81)       | 72 (60–85) | 62 (48–76) | <0.0001  |
| Social role                     | 87.5 (71.88–100) | 87.5 (71.88–100) | 75 (59.38–100) | 0.0891   |
| Pain                            | 70 (24.38–80)    | 78.75 (67.5–90) | 70 (43.13–90) | 0.0013   |
| General Health                  | 55 (47.5–70)     | 75 (63.75–80) | 75 (61.25–81.25) | <0.0001  |

36-Item Short-Form Health Survey (SF-36) assessments were performed pre-intervention, post-intervention, and at a 3-month post-intervention follow-up. Each outcome has its respective median and quartile 1–quartile 3 (Q1–Q3).
that patients with UI do not report their diagnosis to health professionals and normalize the symptomatology, taking years to receive the necessary treatment, making surgery the only viable alternative to their condition [34, 35]. Despite reports showing that more people with UI are consulting physicians, many women remain undiagnosed. [34, 36, 37]. For this reason, preventive and timely intervention in pelvic floor education and rehabilitation is an excellent alternative to reduce associated healthcare costs and positively impact the QoL of patients with UI and pelvic floor dysfunctions [36, 38]. In this sense, it is central to implement a multidisciplinary approach to education, screening, and diagnosis of UI to perform a timely and early low-cost rehabilitation [37–39]. Despite this background, public policies do not align with the available evidence. The national reports obey local and private initiatives due to the interest of healthcare professionals or teachers [14, 40, 41]. Therefore, it is highly relevant to generate evidence that supports massive and public programs with low implementation costs and with a community approach that can have a positive impact on the QoL of users with UI and pelvic floor dysfunctions, as is the case of the “Esencia de Mujer” (Essence of a woman) program.

Our study was able to recruit 22 women with a diagnosis of UI. Although the sample is limited in the number of participants compared to other studies, there are reports with similar individuals that have reported significant changes in QoL and muscle strength after a rehabilitation protocol focused on UI [10]. In a randomized controlled study, Nascimento-Correia et al. recruited 30 women with pelvic floor dysfunction and a diagnosis of UI but divided their sample into control and intervention groups. Thus, the authors performed therapy for 12 weeks during 1 h in group sessions with only 15 women, obtaining significant differences in QoL measured with the King’s health questionnaire (KHQ) [18].

No dropouts were reported among the recruited patients. However, the attendance percentage at scheduled sessions was 80.35 ± 22.95%. We found no reports for this indicator. We believe that the margins of nonattendance could be directly related to the conditions of confinement and quarantine due to the Coronavirus disease 2019 (COVID-19) pandemic in which the study was conducted [42], so it would be necessary to consider implementing this program in a non-pandemic context to evaluate its potential impact.

One of the factors influencing UI and pelvic floor dysfunctions is age. This factor in our sample ranged from 44 to 75 years, averaging 59.5 ± 8.71 years. These age values are similar to those reported in the systematic review by Radzimina et al., where the patients’ ages range from 40 to 85 years [10]. The coincidence is because the prevalence of pelvic floor dysfunctions is mainly seen over the fourth decade of life [43]. However, a group of patients, such as pregnant patients, escapes this reported range. Cohen-Quintana et al. report in a descriptive study including pregnant volunteers with pelvic floor dysfunction and UI, a mean age of 26.65 years [40]. The sum of these antecedents and our results allows us to consider the importance of the transversality of pelvic floor rehabilitation programs throughout the life cycle. At the same time, it must be regarded that the world population is aging, which means that there will be a high percentage of the world population with UI; nonetheless, studies are needed to characterize the current reality of pelvic floor dysfunctions and age.

Another factor influencing the diagnosis of UI and pelvic floor dysfunction is BMI, which in our sample was 27.22 (23.81–35.47) kg/m². This factor becomes relevant since it has been reported, utilizing logistic regression, that overweight and obesity are associated with a higher risk of presenting pelvic floor dysfunctions. This risk ranges from 2.0 to 2.6 times, respectively [44]. Considering this evidence and our results, it is essential to address pelvic floor dysfunctions from a multidisciplinary approach that considers nutritional and physical activity aspects to reduce BMI and indirectly impact this risk factor for pelvic floor dysfunction.

Different studies have established that pregnancy, instrumental delivery, and cesarean
sections increase the prevalence of pelvic floor dysfunctions [44–46]. Our data coincide with this trend since 95.45% of the sample had at least one pregnancy, 63.64% underwent episiotomy, and 13.64% of the users underwent forceps-type instrumentalization. However, some data may be underestimated since they were obtained by self-reporting and not with access to their clinical records.

In our study, we categorized the sample by classifying the type of UI according to the ICS. The most significant predominance in the initial evaluation is stress incontinence, with 54.55%, and none of the patients presented continent. Our sample has similar behavior to those reported in the literature, where stress incontinence is predominant according to the ICS classification [47–49]. At the end of the pelvic floor rehabilitation program, 40.91% of the sample was classified as continent 3 months after the final evaluation, and continent status was maintained in 31.82% of the sample. Although the authors do not use the same follow-up period, they agree that the changes obtained after a rehabilitation program are maintained until at least 6 months post-intervention [48, 49].

As we have seen, QoL is one of the indicators of success in pelvic floor rehabilitation programs for UI. Our study reflected significant changes in the ICIQ-SF questionnaire, a tool designed to identify people with UI and the impact on QoL. Our results show that our kinesic rehabilitation program positively impacts the QoL of users with UI and pelvic floor dysfunctions, maintaining these results up to 3 months after the end of the program. These positive results in QoL align with the strength assessment measured with the Oxford scale, whose initial median was 2 (1–3) points and 3 (3–4) points at the end of treatment. Unfortunately, muscle strength follow-up was not possible due to the context of the COVID-19 pandemic [42].

Rehabilitation programs have been shown to impact strength and QoL in different populations [10, 18, 28–34]. Among the antecedents, we would like to highlight that the ICIQ-SF questionnaire has been used to evaluate QoL in people similar to those covered in our study, as in the case of post-menopausal women with stress UI [50] and older women with stress UI after a community intervention [51]. In the Chilean population, there is a report by Jerez et al. where, after a pelvic floor rehabilitation program in people with UI, they managed to significantly improve QoL and muscle strength using an exercise protocol similar to the one used by us; nevertheless, they did not evaluate QoL with a validated scale such as the ICIQ-SF [14].

Another factor to consider in evaluating UI is the severity of the clinical symptoms. Concerning the Sandvik severity test, our study obtained a decrease in the severity of urinary incontinence maintained after the 3-month post-intervention follow-up. Considering the categorization provided by the Sandvik severity test, we observed in the initial evaluation that 100% of the sample qualified with a degree of severity and that after the community rehabilitation program, 40.91% of the sample qualified as a continent and 31.82% of users remained in the same category after 3 months of intervention. In contrast, 9.09% of the sample presented very severe categorization at the beginning of the program; after the intervention and at follow-up, no user qualified for this categorization. At 3 months post-intervention, there was a high percentage of decrease in severity even with a high percentage of continent users. Our findings suggest that the decline in QoL and UI severity are directly related. These statements are in line with the results reported by Klovning et al., which evaluated the ICIQ-SF and the Sandvik severity test (called the Incontinence Severity Index in their study), establishing a high correlation between the two questionnaires [52].

One factor influencing pelvic floor dysfunctions related to UI is the level of schooling. In our sample, 18.18% had higher education, and 81.82% had secondary education or less. By logistic regression models in the American population, it has been shown that lower schooling was associated with higher prevalence rates for pelvic floor dysfunctions related to UI [53, 54]. These data are consistent with those obtained by Sacomori et al. in a cross-sectional study of the Brazilian population [55]. Saadia found similar
results in the Saudi population concerning the incidence of UI and educational level, associating this higher incidence of cases with the lack of general information on pathology [56]. These antecedents lead us to postulate that the same phenomenon of higher incidence would occur at a lower educational level in our sample. However, due to the nature of our study, we cannot correlate these variables, so other analyses are needed to elucidate this question in the Chilean population.

Finally, and to complement the results obtained in QoL evaluation, we wanted to determine whether the changes observed by a specific UI scale, such as the ICIQ-SF, could also be investigated with a generic QoL scale already validated and applied in the Chilean population, as is the case of the SF-36 [22, 57]. Of the areas considered by the SF-36, we observed: (A) Stabilization of the variable limitations of physical health. (B) Significant improvements were maintained even at the 3-month post-intervention follow-up in the physical functioning and general health sections. (C) Downward trends with no significant differences in the limitations in emotional problems and social roles. (D) Improvements at the end of the intervention but a slight decrease in the Energy/fatigue, emotional role, and pain sections at follow-up. These results clearly show that the pelvic floor rehabilitation intervention for UI successfully impacted the QoL since none of the areas considered by the questionnaire generated a decrease in their score with significant differences. Even two of them were upward even at post-intervention follow-up. Our results agree with a previous report that found improvements in QoL measured with SF-36 after an intervention protocol in postpartum Chinese women who underwent pelvic floor muscle training versus the control group [58].

Concerning the Chilean population and QoL according to the SF-36, it has been seen that UI is associated with lower SF-36 values [34]. Several reports and our data support that the SF-36 is a valid tool for screening and assessing QoL in populations with pelvic floor dysfunction and UI [22, 34, 57, 58].

Regarding the role of the COVID-19 pandemic in QoL, Guzmán-Muñoz et al. compared self-reported QoL in young adults in a non-pandemic setting versus a setting under health alert for COVID-19. They demonstrated in women that Health-Related Quality of Life (HR-QoL) decreased during the COVID-19 quarantine in the dimensions of energy/fatigue, social function, emotional role, mental health, and general health [59]. This result leads us to believe that the confinement factor could have negatively impacted the perception of QoL in our sample. However, other studies are needed to evaluate a pelvic floor rehabilitation program for patients diagnosed with UI without the conditioning factor of the COVID-19 pandemic.

As we highlighted above, the Chilean reality is that few intervention initiatives focus on community pelvic floor rehabilitation despite the available evidence supporting its implementation [10, 18, 28–34]. Our study establishes novel and essential milestones from community rehabilitation that could be replicated: (A) Implementation of the rehabilitation program in a neighborhood unit of the commune of Maipú that managed to detect pelvic floor dysfunctions that were not being treated in the public health network. (B) To focus on a low-cost community intervention on the pelvic floor with positive results that can be implemented with competitive funds that are not usually used in health care. The sum of both factors suggests that this type of program can generate a positive impact at a low cost in the healthcare network and easy access for the community, so it would be essential to consider its massification.

7.6 Conclusions

In the sample studied, improvements in QoL, UI severity, and pelvic floor muscle strength were observed after applying a community kinesic rehabilitation program to patients with pelvic floor dysfunctions and UI diagnosis. Our data align with those reported in the literature, so it is relevant to consider the implementation of
community programs aimed at education, screening, and early rehabilitation of these patients to reduce the prevalence of these clinical conditions.

On the one hand, future studies should consider increasing the sample studied, evaluating control groups, considering other pathologies that entail pelvic floor dysfunctions, and follow-up of the groups for a more extended time, both at the world level and in the Chilean population. On the other hand, whether carrying out this kinesic rehabilitation program outside the pandemic context of COVID-19 would have a more significant effect on the population studied

**Statements and Declarations**

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**Ethical Approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval was granted by the Scientific Ethics Committee of the Universidad de las Américas (Approval number ID: CEC_FP_2021009).

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Part II

Molecular Pathology of Endocrine and Muscular Disorders
Iodine Intake Based on a Survey from a Cohort of Women at Their Third Trimester of Pregnancy from the Bosque County Chile

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Abstract

Adequate iodine nutrition is fundamental for all humans and is critical during pregnancy and lactation due to iodine forms part of the structure of thyroid hormones (THs) and it is required for THs function. Iodine is a scarce micronutrient that must be obtained from the diet. Sufficient iodine can be found in the nature from seafood and given it is not frequently consumed by Chileans, public health policies state that table salt in Chile must be iodized. Health plans must be monitored to determine if the intake of iodine is being appropriated and the population has not fallen in deficiency or excess. The aim of this work was to evaluate iodine intake in 26 women at the third trimester of pregnancy.

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Pregnant women are resident from El Bosque a low-income County located in Santiago de Chile. These Chilean pregnant women were recruited by nutritionist at the Centros de Salud familiar (CESFAM). A 24 h dietary recall (24 h-DR) was applied to them to evaluate iodine intake. Samples of urine and blood were taken by health professionals to analyze parameters of thyroid function and to measure urine iodine concentration (UIC). The survey analysis showed that the iodine consumption in these pregnant women derived mainly from salt, bread and milk and not from seafood. The survey analysis indicated that iodine intake was above the requirements for pregnant women. However, the average UIC indicated that iodine intake was adequate, suggesting the need to find a better parameter to determine iodine intake in pregnant women.

8.1 Introduction

Adequate iodine nutrition is fundamental for all humans, and it is critical during pregnancy and lactation. The main biological function of iodine is to be an essential component of the structure of thyroid hormones (HTs) [1]. Iodine is obtained from the diet as iodide, and it is concentrated at the thyroid gland by the Na⁺/I⁻ symporter (NIS) [2]. About the 70–80% of the iodine is concentrated by the thyroid gland. Iodine absorption occurs principally at upper small intestine where all different forms of iodine are reduced to iodide which is transported by NIS action at the apical surface of the enterocyte. NIS expression is regulated mainly by thyroid stimulating hormone (TSH) and iodine itself. TSH stimulates the synthesis of NIS and its targeting to the basolateral plasma membrane of thyrocytes. Meanwhile, acute high levels of iodine in the blood reduces NIS expression by the thyroid gland, this effect is known as the Wollk-Chaikoff effect. After iodide is being released to the circulation, it is taken up by the thyroid gland and kidney depending on physiological needs [3]. During pregnancy there is an increase in the amount of iodine required for the pregnant woman compared to normal adult. This is due to the maternal thyroid gland must synthesize THs for herself and the fetus at least until 20th week of gestation [2]. It has been reported that at the
beginning of pregnancy not only TSH stimulates the thyroid gland to transport iodide into the thyroid gland and to increase the THs synthesis, but also the human chorionic gonadotropin hormone (hCG). Thus, the maternal thyroid gland will increase iodide clearance and it will be subjected to a physiological challenge that in many women becomes a physiological stress [2]. In fact, it is highly frequent that at the beginning of pregnancy women developed transient hypothyroxinemia (HTX). It has been reported in humans and in animal models that HTX impairs cognition in the offspring. To overcome iodide deficiency in pregnancy, women are encouraged to increase iodine consumption. Unfortunately, iodine is found in very low concentrations at the soil and in vegetables, fruits, and meat which contributes with the prevalence of iodine deficiency worldwide [4]. To overcome this problem, in Chile, since 1960 public health policies had been developed to ensure that the table salt must be iodized to prevent iodine deficiency [5, 6]. However, the high consumption of salt has caused in Chilean population to be exposed to iodine excess intake and consequently high prevalence of thyroid diseases [7]. In Chile about 24% of the adult population suffers of thyroid diseases [8, 9]. Moreover, it has been reported that iodine deficiency or excess during gestation is responsible for growth retardation, short stature, bone malformations and cognitive impairment in the offspring [10, 11]. For these reasons iodine intake in pregnant women needs to be monitored to prevent the consequences of iodine deficiency or excess in the offspring. Therefore, knowing the status of iodine nutrition and thyroid function in pregnant women will be a valuable information for the developing of proper health policies. The assessment of iodine status is not an easy task due to all available biomarkers of nutritional iodine status have their limitations. The most frequently biomarker used index is urinary iodine concentration (UIC) [12]. UIC is a sensitive indicator of iodine intake during the days prior to sampling. However, UIC is not indicative of long-term iodine intake and of the iodine nutritional status. UIC can be applied as a population-based indicator of iodine nutritional status [13]. According to the World Health Organization (WHO), a median population of UIC below 100 µg/L for pregnant women is considered indicative of iodine deficiency. While values between 100 and 150 µg/L reflect normal iodine intake and values above 300 µg/L indicate excessive iodine intake [12]. The determination of THs as a biomarker of iodine intake is less sensitive and is only affected when iodine deficiency is severe [13, 14]. TSH can be used as a biomarker for iodine nutritional status mainly when iodine deficiency is also severe [15]. Tg is a thyroid specific protein and precursor of THs. It has been proposed that the levels of Tg in the blood can be used as an iodine status biomarker. This is because exist a positive relationship with thyroid mass increase [16]. During iodine deficiency Tg levels increase in serum. Importantly, to consider is the interference of anti-Tg antibodies in the serum. These antibodies can bind Tg reducing Tg levels and resulting in an inappropriate estimation of iodine status [17]. Moreover, assay reproducibility and standardization is still lacking in Tg available assays [17, 18]. Thyroid physiological alterations related to iodine intake have a U-shaped distribution [19], therefore, public health strategies focused on guaranteeing an adequate intake of iodine in the population should focus on maintaining levels within an optimal range. Scientific evidence has shown that iodine excess can induce physiological changes in susceptible groups, particularly those previously exposed to iodine deficiency, and pregnant or lactating women [20]. Several expert committees have pointed out the need to identify the most important dietary sources of iodine. Thus, this information will contribute to improve the data on the iodine content in foods and beverages [21]. This information will also allow to understand in more detail the population patterns of iodine intake. Therefore, by applying dietary assessment methods to estimate iodine intake, and thus be able to assess iodine status through dietary recommendations [22]. All food intake assessment methods pose several challenges, as many of the instruments used are time
consuming for participants and rely on memory, portion estimation, and precise frequency. Additionally, dietary methods must rely on accurate and reliable data about the chemical composition of different macronutrients and micronutrients in foods. Moreover, these methods should include native and traditional foods used from each country and region [23]. Regarding the determination of iodine intake, there are additional limitations, as the iodine content of foods can vary considerably between similar products, for example, as in dairy products or between the same species of seafood [24]. Twenty-four-hour dietary recalls (24 h-DR) are one of the most used dietary methods to qualitatively determine the consumption of one or more foods in nutritional research [23]. 24 h-DR can also be used to assess habitual iodine intake. However, all 24 h-DR developed must be validated for the specific nutrient and population that will be used [25]. On the other hand, the success of these methods relies on the recall and accuracy of whom answer the questions. An advantage of this method is that it is very useful for evaluating foods that contain high levels of a specific nutrient [26]. We think that this method can be applied to determine the intake of iodine in pregnant women population given that iodine must be present in high level in frequent type of food like are salt, bread and milk. Hence, the usefulness of this method will be for evaluating the nutritional contribution of bread and dairy products as a primary source of iodine, given their high and cross-sectional level of consumption in the Chilean population. According to the National Health Survey (ENS), practically the entire population reports consuming bread while 98% report consuming some type of dairy products, which corroborates the massiveness of the foods to be evaluated [27]. Several studies have used this strategy to evaluate iodine consumption and its nutritional sources, particularly in countries where iodine consumption is a constant concern, such as Spain [28], Norway [29, 30], Denmark [31], Poland [32], Australia [33], and England [34, 35]. In Latin America, some studies have been published on the Brazilian population [36]. In Chile there are no studies on the consumption of foods that contribute to iodine nutrition. The aim of this work is to determine the status of iodine nutrition in a cohort of Chilean pregnant women from El Bosque County, a low-income municipality located in Santiago de Chile. For that 24 h-DR was performed and the survey was analyzed by using a Food Processor Software. The iodine content in bread and milk that was used in this study derived from our data. This is the first work that compares iodine intake in Chilean pregnant women based in a 24 h-DR with UIC.

8.2 Methods

Study design and participants: Pregnant women at the third trimester of gestation were recruited at primary health care center from the following Centros de Salud Familiar (CESFAM) of El Bosque County: Carlos Lorca, Cóndores de Chile, Mario Salcedo, Orlando Letelier and Santa Laura from the Servicio de Salud Metropolitano Sur (SSMS). El Bosque is a Municipality located in the southern part of Santiago. According to the 2017 Census, the Municipality is home to 162,505 inhabitants (National Institute of Statistics) [37]. El Bosque has higher rates of poverty than the Metropolitan Region and National averages (14%, 11.1% and 13.7% respectively), as well as higher enrolment in the National Health Fund or public insurance (88.4%, 70% and 76.5%) (Ministry of Social Development) [38]. The inclusion criteria used in this study are shown in Table 8.1. Briefly, adult (> 18 years old) and single pregnancy women were included. Pregnant women that were recruited signed an informed consent and responded a brief health questionnaire to inform about their gestational age, parity, gravidity, weight, height, presence thyroid disease or other disease (chronic or not), daily medication, smoking habit, type, and frequency of intake of multivitamins with or without iodine supplementation. Five mL of blood were taken for measuring thyroid hormones,
TSH and thyroglobulin (Tg). Three to five mL of urine sample were taken for iodine and creatinine determination. Both blood and urine were obtained by health professionals that belong to the CESFAMs. Urine and plasma samples were stored at −20 °C until analyses. This study was approved by the ethical committee of the Servicio de Salud Metropolitano Sur (SSMS) (see ethical approval section).

**UIC determination:** Urine iodine concentration (UIC) was measured in duplicate at the Laboratorio de Endocrinología e Inmunología from Universidad Andrés Bello by using a modification of the Sandell-Kolthoff reaction with spectrophotometric detection (Epoch, Biotek) and with a method sensitivity of 12 μg /L [39]. Urine samples were frozen and kept at -20 °C until analysis. Briefly, the samples were mineralized by adding ammonium persulfate and homogenized by vortexing at 95°C for 40 min followed by cooling bath at 20–30 min. 50 μL of sample or standard were added to each well of a 96 well plate followed by a solution of arsenic acid and agitated for 1 min. After agitation, a solution of Cerium Ammonium (IV) sulfate was added and incubated for 30 min at room temperature. Then, the absorbance at a 450 nm wavelength was determined using a spectrophotometer. The values were interpolated from a standard curve ranging from 0 to 50 μg/dL of iodine.

**Laboratory analyses:** Creatinine (Cr) (mg/dL) in urine samples and free T₃ (fT₃) (pg/mL), total T₃ (tT₃) (ng/dL), free T₄ (fT₄) (ng/dL), total T₄ (tT₄) (μg/dL), thyroid stimulating hormone (TSH) (μUI/mL) and thyroglobulin (Tg) (ng/mL) in serum samples were measured in a certified laboratory (IEMA, Providencia, Santiago). Hormones and Tg were measured by chemiluminescence using a Maglumi 2000 and Immulite 2000 equipment respectively. Cr was measured using a BA400 equipment (IEMA, Providencia, Santiago).

**24 h dietary recall (24 h-DR):** A standard set of questionnaires were designed to collect basic information from participants. This information included health status, dietary information due to allergies, intolerances, food preferences, use of dietary supplements, salt, and condiments consumption. Experienced dieticians performed a 24 h-DR by phone followed by a food portion size estimation. The 24-h diet records included food or drink. Details of the food such as brand, preparation/cooking method, and weight at the time of consumption were registered. To analyze food preparations, the weight of all individual ingredients and the weight of the portion were registered for all the preparation consumed by the patient [40]. Discretionary salt intake, which correspond to salt added during cooking or at the table, was calculated according to standard referent portion sizes as pieces and/or spoons. The amount of iodine in salt was considered as 40 μg/g according to the last modification of the Chilean salt ionization program [5]. The iodine content in milk skim, whole milk, lactose-free milk, powdered milk, and traditional Chilean white bread (marraqueta/hallulla) was determined by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (DICTUC). The values obtained by ICP-MS for bread and milk were included in the present study for the calculation of iodine intake. The iodine content values available in the United States Department of Agriculture (USDA) food composition database [41] were used as reference values for the rest of foods. Taking together all this data, the theoretical estimated iodine consumption (tEIC) was
computed using the ESHA Food Processor software (version 11.11.32; 2022) by a trained dietician.

**Determination of iodine concentration in milk and bread by ICP-MS**: Iodine content in whole milk, lactose-free whole milk, semi-skimmed liquid milk, liquid skimmed milk, lactose-free liquid skimmed milk, whole milk powder, powdered skim milk from Colón brand and bread white samples (and white traditional Chilean bread named hallula and marraqueta) by using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) briefly, iodine measurement were performed by the Norma ISO 20647:2015. First, a sample of bread or milk is digested in KOH at 105 ± 5 °C during. Then the iodine is stabilized in NH₄OH and Na₂S₂SO₃. Then, the samples were reconstituted and filtered. The filtrated sample was analyzed directly at the ICP-MS using praseodymium as internal standard. The quantification was performed through a calibration curve which followed the laboratory requirements: the error for each point was established as < 10% with an r value < 0.998. The set of samples plus blanks, were analyzed by duplicate and the reference material fulfill the acceptance criteria established as: Blank < LD, RSD < 10% with a recuperation of reference material between 90 and 110%. The ICP-MS equipment is located at the Dirección de Investigaciones Científicas y Tecnológicas de la Pontificia Universidad Católica de Chile, (DICTUC) in Santiago, Chile. These experimental values were used to determine the calculated iodine consumption (cIC) for each patient according to the data provided by the patient when interviewed by the dietician in the 24 h-RD.

**Statistical analysis**: Statistical analyses were conducted using the statistical functions in Python SciPy packages (version 1.9.9; available from: https://www.scipy.org/). All plots were drawn using Python Matplotlib package (version 3.6.0; available from: https://matplotlib.org/) and GraphPad Prism 9 (version 9.4.1). Student’s T test and Spearman’s or Pearson’s correlation coefficient (r) was calculated. Correlation will be considered as follows according to r value: 0–0.1 as negligible correlation; 0.10–0.39 as weak correlation; 0.4–0.69 as moderate correlation; 0.7–0.89 a strong correlation and 0.9–1 a very strong correlation according to the criteria proposed by Schober et al., [42]. Standardized values ((Value-Median)/SD) were used to obtain the histogram plots and scatter plots. A Locally Weighted Scatterplot Smoothing (LOWESS) representative curve was added. LOWESS smoothing was applied to show the trends for each variable pair (light red) and each point was colored according to the patient iodine consumption. P values were considered statically significant when *p < 0.05, **p < 0.01 and ***p < 0.001. Principal components analysis (PCA) was performed using a standardizing method and selecting the principal components by the “Kaiser rule” with eigenvalues > 1. Table S4 shows the PC summary for all PCs generated. PC1 and PC2 account for the 88% of the cumulative variance of the analyzed population.

### 8.3 Results

#### 8.3.1 Characteristic of Pregnant Women that Participated in the Study

A total number of 72 pregnant women were recruited for this study. From them, only 26 women fulfilled the inclusion criteria. A 88.45% was recruited at CESFAM Carlos Lorca meanwhile a 7.69% and a 3.8% of all the patients were recruited at CESFAM Cóndores de Chile, and CESFAM Mario Salcedo respectively (Table 8.2). The characteristics of the recruited pregnant women are shown at Table 8.3. All patients were residents from El Bosque that have

| Primary health service          | Percentage | Count |
|---------------------------------|------------|-------|
| CESFAM Carlos Lorca             | 88.45%     | 23    |
| CESFAM Cóndores de Chile         | 7.69%      | 2     |
| CESFAM Mario Salcedo            | 3.8%       | 1     |
an average age of 30.4 years old and average of gestational age of 23 weeks at the time they were recruited for this study. The survey, urine and blood samples were obtained at the third gestation trimester by health professionals. Anthropometrical data showed an average weight of 76.86 Kg and an average height of 159.8 cm. The calculated BMI value for this group of pregnant women was of 30.03. This BMI is considered obese according to WHO guidelines [43]. In fact, a 46% of the participants were considered obese and only a 11% showed a healthy weight. A 92.3% of patients were non-smokers with a very low percentage of chronic diseases as diabetes or hypertension (Table 8.4). Regarding vitamins and supplements consumption the 65.4% of the interviewed women were consuming iron meanwhile the 23.1% were consuming folic acid as prenatal supplements (Table 8.5).

### 8.3.2 Thyroid Physiological Parameters

To evaluate thyroid function in pregnant women, thyroid physiological parameters were measured. The levels of fT3, rT3, rT4, fT4, TSH and Tg were determined from blood samples and Cr and UIC were determine in urine samples. The global mean and S.E.M for each parameter can be found at Table 8.6. The mean of all thyroid physiological parameters was observed between the normal ranges for pregnant women [44–47].

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**Table 8.3** Characteristics of pregnant women included in the study

| Characteristic                           | Value          |
|-----------------------------------------|----------------|
| Number of total Pregnant women included | 26             |
| El Bosque resident patients             | 100% (26)      |
| Average age (years ± SEM)               | 30.4 ± 1.1     |
| Average gestational age (weeks ± SEM)   | 22.8 ± 1.2     |
| Average weight (Kg ± SEM)               | 76.9 ± 2.9     |
| Average height (cm ± SEM)               | 159.8 ± 1.2    |
| Average BMI (Kg/m² ± SEM)               | 30.0 ± 1.2     |
| Average gravidity (number ± SEM)        | 1.9 ± 0.2      |
| Average parity (number ± SEM)           | 0.8 ± 0.2      |

**Table 8.4** Health status of pregnant women included in the study

| Status                                | Percentage |
|---------------------------------------|------------|
| Smoking                               | 7.7% (2)   |
| Non-smoking                           | 92.3% (24) |
| Underweight patients (BMI < 18.5)     | 3.8% (1)   |
| Healthy weight patients (18.5 < BMI < 24.9) | 11.5% (3) |
| Overweight patients (25 < BMI < 29.9)  | 30.8% (8)  |
| Obese patients (30 < BMI < 39.9)      | 46.2% (12) |
| Extremely Obese (BMI > 40)            | 3.8% (1)   |
| Insulin resistant                     | 7.7% (2)   |
| Diabetic                              | 7.7% (2)   |
| Hypertensive                          | 3.8% (1)   |
| Hypothyroid                           | 3.8% (1)   |
| Others (Asma. Ulcerative colitis. Hyperemesis. Headache. depression) | 7.7% (2) |

In parenthesis the number of women with these characteristics is indicated.
Table 8.5 Information of supplement and vitamin consumption taken for pregnant women included in this study

| Supplements |  |
|-------------|---|
| Iron        | 65.4% (17) |
| Folic acid  | 23.1% (6)  |
| Calcium     | 7.7% (2)   |
| Omega 3     | 3.8% (4)   |

| Vitamins |  |
|----------|---|
| Prenatal vitamins | 15.4% (8) |

In parenthesis the number of women with these characteristics is indicated

Table 8.6 Average for thyroid physiological parameters

| Parameter                  | Value (± SEM) | Normal Range |
|----------------------------|---------------|--------------|
| Creatinuria (mg/dL ± SEM)  | 90.03 ± 11.72 | (90–300 mg/dL) |
| Free T₃ (pg/mL ± SEM)       | 3.188 ± 0.053 | (2.5–13 pg/mL) |
| Total T₃ (ng/dL ± SEM)      | 170 ± 9.70    | (99–257 ng/dL) [40] |
| Total T₄ (µg/dL ± SEM)      | 10.98 ± 0.32  | (7.3–15.1 µg/dL) [41] |
| Free T₄ (ng/dL ± SEM)       | 0.994 ± 0.017 | (0.5–4.82 ng/dL) [41] |
| TSH (µUI/mL ± SEM)          | 2.025 ± 0.19  | (0.3–3.5 µUI/mL) [42] |
| Thyroglobulin (ng/mL ± SEM) | 17.83 ± 4.88  | (5.3–25.2 ng/mL) [43] |
| UIC (µg/L ± SEM)            | 212.6 ± 19.84 | (150–249 µg/L) [5] |
| I/Cr (µg/g ± SEM)           | 304.8 ± 34.76 | (33–535 µg/g) [b] |

[a] National Health Survey (ENS). Chile 2017
[b] Urinary iodine percentile ranges in the United States 2013 (normal ranges)

Individual analysis of thyroid physiological parameters for each patient are shown in Table S1. It was observed values in the normal range for fT₃, fT₄ and Tg. However, one patient had high levels of fT₃, another patient had low levels of fT₃, and one patient had high level of tT₄. Meanwhile, two patients had high levels of TSH and other two patients presented high levels of Tg. Regarding, to the individual values of UIC six patients presented a UIC value over the normal range and three patients had low levels of UIC. The values of UIC were corrected by Cr and the analysis showed that four patients had higher levels of UIC/Cr. Correlation analysis showed the absence of strong associations between thyroid physiological parameters (Fig. 8.2).

8.3.3 Food Iodine Content and Estimated Iodine Consumption

Iodine concentration in food varies greatly between regions because it depends on the soil and high levels of iodine are found in areas that are close to the sea [10, 48, 49]. In Chile the salt is iodized, and it has been shown that the population ingest 10 g of salt daily [50]. Chilean diet has a higher consumption of bread, milk, and salt [50]. Therefore, iodine concentration was measured for two types of highly consumed breads in the Chilean population and different types of milk available in the Chilean market by ICP-MS (see methodology). This methodology presents an increased selectivity and sensitivity for iodine.
determination [51]. The obtained results are shown in Table 8.7, interestingly we found that semi-skimmed liquid milk (Colún), lactose-free semi-skimmed liquid milk (Colún) and lactose-free skimmed liquid milk (Colún) have high concentrations of iodine 92.40 µg/100 mL, 91.50 µg/100 mL and 87.40 µg/100 mL respectively compared to the other types of milk subtypes (Table 8.7). Moreover, hallulla has the higher iodine concentration compared to marraqueta 22.98 µg/100 g and 8.49 µg/100 g respectively.

8.3.4 Theoretical Estimated Iodine Consumption (tEIC) and Calculated Iodine Consumption (cIC)

The theoretical estimated iodine consumption (tEIC) for each pregnant woman of this study was obtained from the analysis of a 24 h-DR by trained nutritionists using the Food processor software. The data from the 24 h-DR was entered into the Food Processor Software to obtain the tEIC. The value of iodine used for bread and milk in the analysis with the Food Processor Software corresponded to the value obtained from the ICP-MS determination (Table 8.7), meanwhile for any other food type the iodine value was the one provided the software manufacturer. The reason for that lies in that bread and milk are frequent consumed by pregnant women and the content of iodine in these types of foods varies greatly in Chile compared to other countries [50] (Table S3). The tEIC for each patient is shown in Table S3. Based on the recommended dietary allowance (RDA) the patient’s daily iodine dietary intake was classified as insufficient (<160 µg), adequate (160–220 µg) above the requirements (220–220–299 µg) or excessive (> 300 µg). Interestingly, the distribution of tEIC in this group of patients was 3.84% for those with insufficient iodine intake, 26.9% was under adequate iodine intake, 38.5% was above requirement and 30.8% under excessive iodine intake with a mean of tEIC for this group of pregnant women above requirements. Considering that the population residing at El Bosque County, is considered as a low-income population and that they consume higher amounts of bread, milk and salt, we estimated a calculated iodine consumption (cIC) based on the amounts of these foods consumed by the patients. The cIC considered the iodide ingested daily only due to bread, milk and salt daily consumed provided by the patient in the 24 h-DR and using the ICP-MS data previously obtained. The individual cIC for each pregnant woman and the mean plus SEM for the study group are shown in Table S2. At the Table 8.8, cIC can be compared to tEIC. The obtained tEIC was of 269.6 ± 16.40 µg/day meanwhile the cIC was of 222.6 ± 16.64 µg/day. Figure 8.1 shows the comparison between tEIC and cIC. The statistical analysis showed that there is not significant difference between tEIC and cIC (Fig. 8.1a).

Table 8.7  Iodine content in Chilean bread (µg/100 mg) and milk (µg/100 mL or µg/100 g) measured by ICP-MS

| Type of Product                  | Iodine Content (µg/100 mg or µg/100 g) |
|---------------------------------|---------------------------------------|
| Whole liquid milk (Colún)       | 22.60                                  |
| Lactose free whole liquid milk (Colún) | 22.40                                  |
| Semi-skimmed liquid milk (Colún) | 92.40                                  |
| Lactose-free semi-skimmed liquid milk (Colún) | 91.50                                  |
| Skimmed liquid milk (Colún)     | 34.80                                  |
| Lactose-free skimmed liquid milk (Colún) | 87.40                                  |
| Whole powdered milk (Colún)     | 50.30                                  |
| Skimmed powdered milk (Colún)   | 42.40                                  |
| Marraqueta bread                | 8.49                                   |
| Hallulla bread                  | 22.98                                  |
and the correlation analysis showed a strong correlation between both parameters $t_{EIC}$ and $c_{IC}$ (Fig. 8.1b) suggesting that salt, bread, and milk could be the principal types of food contributing with the daily dietary iodide intake of the Chilean pregnant women. A PCA analysis was performed over the variables contributing to $t_{EIC}$ to understand their correlation and contribution to this parameter. Figure 8.1c shows the obtained loading plot. Here we observed the clustering of bread and milk showing a strong correlation between both variables with a moderate correlation with PC1 and PC2. Fish and seafoods presents a moderate correlation with PC1 and week correlation with PC2, on the other hand meat, present negligible correlation with both PC1 and PC2 meanwhile fruits and vegetables present a very strong negative correlation with PC2. Interestingly, there is cluster composed by snacks, rice and noodles and butter/ham/cheese showing

| Patient | $t_{EIC}$ (µg/day) | Classification according reference value* (160–220 µg/day) | $c_{IC}$ (µg/day) | Classification according reference value* (160–220 µg/day) |
|---------|-------------------|----------------------------------------------------------|------------------|----------------------------------------------------------|
| 1       | 378.71            | Excessive                                                | 232.17           | Above requirements                                        |
| 2       | 423.01            | Excessive                                                | 358.76           | Excessive                                                |
| 3       | 167.96            | Adequate                                                 | 100.24           | Insufficient                                             |
| 4       | 270.50            | Above requirements                                       | 149.33           | Insufficient                                             |
| 5       | 253.57            | Above requirements                                       | 171.52           | Adequate                                                 |
| 6       | 312.15            | Excessive                                                | 271.79           | Above requirements                                        |
| 7       | 162.63            | Adequate                                                 | 109.20           | Insufficient                                             |
| 8       | 124.84            | Insufficient                                             | 79.40            | Insufficient                                             |
| 9       | 164.08            | Adequate                                                 | 154.83           | Insufficient                                             |
| 10      | 263.50            | Above requirements                                       | 225.12           | Above requirements                                        |
| 11      | 249.51            | Above requirements                                       | 222.15           | Above requirements                                        |
| 12      | 261.95            | Above requirements                                       | 214.49           | Adequate                                                 |
| 13      | 259.81            | Above requirements                                       | 223.78           | Excessive                                                |
| 14      | 190.39            | Adequate                                                 | 183.68           | Adequate                                                 |
| 15      | 295.25            | Above requirements                                       | 242.87           | Above requirements                                        |
| 16      | 254.83            | Above requirements                                       | 167.47           | Adequate                                                 |
| 17      | 332.42            | Excessive                                                | 303.97           | Excessive                                                |
| 18      | 265.78            | Above requirements                                       | 221.22           | Adequate                                                 |
| 19      | 384.51            | Excessive                                                | 362.35           | Excessive                                                |
| 20      | 436.99            | Excessive                                                | 406.67           | Excessive                                                |
| 21      | 380.34            | Excessive                                                | 345.28           | Excessive                                                |
| 22      | 190.10            | Adequate                                                 | 129.23           | Insufficient                                             |
| 23      | 310.29            | Excessive                                                | 285.26           | Above requirements                                        |
| 24      | 241.86            | Above requirements                                       | 190.49           | Adequate                                                 |
| 25      | 207.72            | Adequate                                                 | 172.98           | Adequate                                                 |
| 26      | 292.00            | Above requirements                                       | 264.16           | Above requirements                                        |
| Mean ± SEM | 269.6 ± 16.40 | Above requirements                                       | 222.6 ± 16.64 | Above requirements                                        |

*NIH Recommended Dietary Allowances (RDAs) for Iodine. Insufficient < 160 µg/day; Adequate 160–220 µg/day; Above requirements 220–299 µg/day; Excessive > 300 µg/day
positive correlation between them and a strong correlation with PC1 but moderate correlation with PC2. Interestingly, salt and eggs does not cluster with any variable presenting opposite directions showing no correlation between them. Nevertheless, salt is clustering with tEIC and cIC which is showing a positive strong correlation between them, with a lesser angle with cIC suggesting a very strong positive correlation between salt and cIC.

8.3.5 Matrix Correlation Analysis

To evaluate the correlation between tEIC, clinical and anthropometric parameters, we perform a correlation analysis matrix. The obtained results are shown in Fig. 8.2. No or weak correlation was observed between tEIC and parameters. A moderate correlation was found between fT3 and fT3 and between fT3 and fT4. Moreover, a moderate correlation was observed Cr with UIC.
and I/Cr. Interestingly, a moderate correlation was observed between tT₃ and age, on other hand tT₄ present a moderate correlation with Tg and height. As expected, a strong correlation was observed between weight and BMI. These results are shown in Fig. 8.2.

8.4 Discussion

Proper thyroid function and iodide consumption during pregnancy are essential aspects for fetus development [52]. There is scarce information regarding thyroid function and iodide intake for Chilean pregnant women and this information is necessary to take proper public health decisions. Specially, in Chile where the prevalence of thyroid diseases is ten times higher than most countries. This study contributes with information regarding iodine consumption and the types of foods that contribute to iodine intake in a cohort of pregnant women from Chile. Our data shows that the intake of iodine based on tEIC and cIC is above requirement, and in contrast the UIC and UIC/Cr values fall into adequate. Moreover, this study shows that milk and bread are the type of food besides salt that contributes better to the intake of iodine in the diet of Chilean pregnant women. The iodine concentration in bread and milk determined in this study used ICP-MS, a very sensitive technique [53–55]. The 24 h-DR revealed that bread and milk are the types of food most consume by pregnant women from this study. Therefore these foods were chosen for the
analysis. The content of iodine for whole milk was 22.60 µg/100 mL which is similar to the value reported using the same technique by United States (~ 22.2 µg/100 mL) [55]. Interestingly, the iodine content in semi and skimmed milks was higher than in whole milks (Table 8.7). The highest value was 92.4 µg/100 mL in the semi-skimmed liquid milk and 42.40 µg/100 g for powdered skimmed milk. It has been reported higher values of iodine content by using ICP-MS. Similar to our determination are the case reported from Finland and New Zealand that indicated 540 µg/Kg [48] and 40–150 µg/Kg [49] respectively of iodine content in skimmed milk. Tinggi et al., reported 3.48 mg/Kg of iodine in non-fat milk [55] a higher content of iodide that we found. Like Tinggi et al., Todorov et al., reported higher values of iodine in non-fat milk (342 µg/100 g) by using ICP-MS. This information is very relevant for chilean public health medicine to aware that pregnant women consuming skim milk should reduce the salt intake to avoid high iodine ingestion [53, 56]. Dahl et al., reported 23, 2 µg/100 mL of iodine content in non-fat milk using ICP-MS [57]. The reason for these variations on iodide concentration in milk between different countries and between whole and skimmed milk are unknown. Factors like the region, manufacture procedures and period of the year could be interesting possibilities to analyze for the variations on iodine content in milk. We found that the content of iodine in Chilean bread (marraqueta) was 8.49 µg/100 g this value indicated that the amount of iodine in Chilean bread was lower than the reported for Leiva et al., in 2002. In fact, they reported 760 µg/g for hallulla and 830 µg/g for marraqueta [6]. The reduction of iodine content in bread can be due to the modification of the iodization health plan in Chile aimed to reduce the intake of iodine [5]. In fact, the iodine content in 8.49 µg/100 g for marraqueta and 22.98 µg/100 g for hallulla were similar to 5.6 µg/100 g reported by Chilean Iodine Educational Bureau in 1952, before the startup of iodization health plan in Chile [58]. In this work, based on the analysis of the 24 h-DR the tEIC applied to pregnant women we found that this population has an iodine intake above the recommended dietary based on the range given by NIH Recommended Dietary Allowances (RDAs) for iodine [59]. The same conclusion was obtained if we only considered the iodine intake due to the consumption of bread, milk, and salt (cIC, Table S2). Both tEIC and cIC values fall above the requirements established for proper iodine intake during pregnancy (Table 8.8 and Fig. 8.1). In fact, no significant differences were observed between tEIC and cIC (Fig. 8.1a). Moreover, a strong correlation was found among these values (Fig. 8.1b), suggesting cIC can be used as a fairly approximation to determine the daily intake of iodine in pregnant women population. Interestingly, iodine content from bread, milk, and salt (cIC) accounted for the ~ 80% of tEIC, indicating that these foods are the main contributors to the daily iodine intake in our group of pregnant women. Based on Fig. 8.1c we would like to emphasize that the values of iodine in both tEIC and cIC correlated better with iodine in salt, milk, and bread than with the value of iodine in seafood. This observation is very relevant because seafood has high iodine content, suggesting that this group has low consumption of seafood. UIC and UIC/Cr were evaluated in the same group of pregnant women that the survey was applied (Table 8.6). The average values obtained for UIC and UIC/Cr indicated that this group of pregnant women had an adequate of iodine intake (Table 8.6). However, we did not find correlation between tEIC or cIC and UIC or UIC/Cr (Fig. 8.2). Mainly, all pregnant women studied in this work showed normal levels of thyroid hormones, TSH and Tg and one or two cases showed values over or lower the normal range (Table S1) [44–46]. Based on Fig. 8.2 UIC or UIC/Cr did not correlate with thyroid hormones or TSH suggesting that THs and TSH does not reflex or correlate with UIC or UIC/Cr. Therefore, we emphasize the necessity to search for better parameters to follow thyroid function and iodine intake individually in pregnant women. This study recruited a small cohort of pregnant women that have in common that they are resident of the same Chilean County, and they were at the same
gestational age when the survey and biological samples were taken. Therefore, our conclusions can only be interpreted for this group and cannot be extrapolated to other Chilean pregnant groups. However, our study indicate that it is necessary to analyze the iodine content in the food and to measure thyroid parameters like thyroid hormones, TSH, Tg, UIC and UIC/Cr to obtain more representative date for Chilean pregnant women. Specially, all these information is important because in Chile the prevalence of thyroid disease is around 24% [5, 27].

8.5 Conclusion

In this work we reported the iodine intake obtained from a 24 h-DR survey in a small cohort of pregnant women was above requirements. These women are from the same Chilean county and were at the same gestational age. The average of cEIC fall above requirements based on the range of proper iodine intake given by RDA. Our data support that the iodine consumption in these pregnant women derived mainly from salt, bread, and milk and not from seafood or other types of food analyzed in the 24-h-DR. This is the first study that incorporates the content of iodine for Chilean bread and milk determined using the ICP-MS, a very sensitive technique for iodine determination. This determination showed that the intake of iodine is above requirements in a low-income population. Even though, cIC did not correlate with the UIC of these pregnant women and the UIC falls in inadequate iodine consumption, the above presented results emphasize the need to find a better parameter and to combine different methodologies to assess iodine intake in pregnant women.

Statements and Declarations

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Disclosure of Interests All authors declare they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval was granted by the Ethical Committee at the Servicio Metropolitano de Salud Sur (SSMS) MEMORANDUM No: 070/2020.

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Appraisal of the Neuroprotective Effect of Dexmedetomidine: A Meta-Analysis

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Abstract

Dexmedetomidine is an adrenergic receptor agonist that has been regarded as neuroprotective in several studies without an objective measure to it. Thus, the aim of this meta-analysis was to analyze and quantify the current evidence for the neuroprotective effects of dexmedetomidine in animals. The search was performed by querying the National Library of Medicine. Studies were included based on their language, significance of their results, and complete availability of data on animal characteristics and interventions. Risk of bias was assessed using SYRCLE’s risk of bias tool and certainty was assessed using the ARRIVE Guidelines 2.0. Synthesis was performed by calculating pooled standardized mean difference and presented in forest plots and tables. The number of eligible records included per outcome is the following: 22 for IL-1β, 13 for IL-6, 19 for apoptosis, 7 for oxidative stress, 7 for Escape Latency, and 4 for Platform Crossings. At the cellular level, dexmedetomidine was found protective against production of IL-1β (standardized mean difference (SMD) = −4.3 [−4.8; −3.7]) and IL-6 (SMD = −5.6 [−6.7; −4.6]), apoptosis (measured through TUNEL, SMD = −6.0 [−6.8; −4.6]), and oxidative stress (measured as MDA production, SMD = −2.0

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(−2.4; −1.4)) exclusively in the central nervous system. At the organism level, dexmedetomidine improved behavioral outcomes measuring escape latency (SMD = −2.4 [−3.3; −1.6]) and number of platform crossings (SMD = 9.1 [−6.8; −11.5]). No eligible study had high risk of bias and certainty was satisfactory for reproducibility in all cases. This meta-analysis highlights the complexity of adrenergic stimulation and sheds light into the mechanisms potentiated by dexmedetomidine, which could be exploited for improving current neuroprotective formulations.

Keywords
Adrenergic · Dexmedetomidine · Neuroprotection · Nervous system · Inflammation · Oxidative stress

Abbreviations
AR Adrenergic receptor
ARRIVE Animal Research: Reporting of In Vivo Experiments
cAMP Cyclic adenosine monophosphate
CI Confidence interval
CNS Central nervous system
DAG Diacylglycerol
DRG Dorsal root ganglion
GIRKs G protein-coupled inwardly-rectifying K⁺ channels
MDA Malondialdehyde
NMDAR NMDA-type glutamatergic receptors
OXPHOS Oxidative phosphorylation
OSP Outlier, significance, and precision
PKA Protein kinase A
PLC Phospholipase C
PIP2 Phosphatidylinositol 4,5-bisphosphate
PKC Protein kinase C
PRISMA Preferred Reporting Items for Systematic reviews and Meta-Analyses
ROS Reactive oxygen species
SMD Standardized mean difference
SYRCLE SYstematic Review Centre for Laboratory animal Experimentation
TUNEL Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling

9.1 Introduction
The preservation of normal physiological functions after insult has been pursued by physicians for centuries. From Hippocrates advising to use snow and ice to reduce hemorrhage in wounded soldiers around 400 BCE, to the first scientific records of the use of hypothermia to preserve physiological functions after traumatic brain injury, asphyxia, and cardiac arrest, strides to regain wellness have been a driving motive throughout history [1]. When presented with a physiological insult, the holistic conception of a human subsides to a more equivocal one carrying an implicit hierarchy with cognitive functions at the pinnacle [2]. Ergo, improving neurological outcomes after biological, physical, or chemical insult becomes pivotal for individual-wide homeostasis. When the limit of adaptive neural responses to injurious agents (e.g., pathogenic bacteria, radiation), stress (e.g., sleep deprivation, traumatic injury, spinal injury, surgery), or deprivation of blood nutrient supply (e.g., during ischemia or hemorrhage) is exceeded, irreversible injury ensues and alternatives for cell fate are (canonically) necrosis or apoptosis. During the onset of membrane damage, main sources of cell stressors include mitochondria (releasing reactive oxygen species [ROS], useful for oxidative phosphorylation [OXPHOS] and the electron transport chain [3]), unconfined lysosomal enzymes, and release of misfolded proteins, activating pro-apoptotic proteins [4], all contributing synergistically to create a recursive pro-inflammatory environment compromising neural function [5] and increasing the risk of mortality [6, 7]. Thus, as long as there are human
activities carrying an implicit chance for neural injury, multivariate, rigorous, and objective appraisal of the effects of novel compounds for neuroprotection will remain a critical need.

Different types of \( \alpha_2 \) adrenergic isoreceptors have been described throughout the body. While \( \alpha_{2B} \) adrenergic receptor (AR) is present and characteristic of peripheral vascular smooth muscle [8], \( \alpha_{2A} \text{AR} \) and \( \alpha_{2C} \text{AR} \) are widely distributed throughout the central nervous system (CNS) [9, 10], of which, the predominant type is the \( \alpha_{2A} \text{AR} \) [11], where it controls adrenergic neuronal exocytosis [12], mediates sedation, analgesia, and sympathetic tone [13, 14].

Dexmedetomidine, an imidazole compound [15], can bind plasma proteins, cross the blood–brain barrier [16], and effectively interact with \( \alpha_{2A} \text{AR} \), which has been described as the predominant isoreceptor mediating the antinociceptive, sedative, and hypothermic actions of dexmedetomidine in the CNS [13]. Intracellularly, stimulation of \( \alpha_{2A} \text{AR} \) by dexmedetomidine leads to the activation of heterotrimeric G \(_i\) \( \alpha \) subunit, inhibition of adenyl cyclase, decrease in cyclic adenosine monophosphate (cAMP) formation, and the preservation of protein kinase A (PKA) resting state [17]. In synchrony, activation of G\(_{i\alpha}\) subunit leads to uncoupling and release of the G\(_i\)-associated G\(_{\beta\gamma}\) subunit, which binds and opens G protein-coupled inwardly-rectifying K\(^+\) channels (GIRKs), increasing K\(^+\) permeability, hyperpolarizing the cell membrane, and decreasing excitability of neurons [18]. Additionally, activation of the G\(_i\)-associated G\(_{\beta\gamma}\) subunit directly inhibits Ca\(_{\text{v}2}\) channels, thus modulating neurotransmitter and hormone release [19]. Moreover, \( \alpha_{2A} \text{AR} \) G\(_i\)-associated G\(_{\beta\gamma}\) subunit leads to phospholipase C (PLC) activation, cleavage of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), increased [Ca\(^{2+}\)], calmodulin activation, and Src-dependent phosphorylation of Erk1/2 [20, 21], as well as the activation of protein kinase C (PKC) after PLC release of diacylglycerol (DAG) in the presence of increased [Ca\(^{2+}\)] [22].

Experimental evidence of dexmedetomidine as a neuroprotective agent has been documented in vivo at both the cellular (protecting against apoptosis, the production of ROS, and increased levels of pro-inflammatory cytokines) and full organism level (preventing neurocognitive deterioration [23, 24] and mortality [25]). For its neuroprotective effects, several molecular mechanisms have been proposed in a wide combination of noxious conditions, among which dexmedetomidine has been reported to significantly reduce levels of hippocampal pro-apoptotic proteins Cyt-c, Apaf-1, and caspase-3 [26], to regulate the p38/c-Myc/CLIC4 signaling pathway leading to apoptosis in the hippocampus [27], to inhibit apoptosis via the HDAC5/NPAS4/MDM2/PSD-95 axis [28], to reduce IL-6 levels in the cortex via pJAK2 and pSTAT3 dependent routes [29], to inhibit ROS production in dorsal root ganglion (DRG) neurons via intracellular signaling routes involving Bax and Bcl-2 [30], and to limit ROS generation via t-BHP [31]. Regardless of the countless independent observations about the underlying molecular mechanism, a quantitative and comprehensive approach regarding neuroprotection exerted by dexmedetomidine at the organism level is yet to be presented. Therefore, the aim of this meta-analysis was to analyze and quantify the current evidence for the neuroprotective effects of dexmedetomidine. To this end, outcomes of inflammation, apoptosis, and oxidative stress, measured in central nerve tissue were sought, as well as outcomes of neurocognitive function.

9.2 Methods

This meta-analysis was conducted following the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) 2020 Guideline [32].

9.2.1 Eligibility Criteria

Synthesis was carried out individually, per outcome. Outcomes of inflammation, apoptosis, and oxidative stress were operationally defined as IL-1\(\beta\), IL-6, apoptosis rate (measured using the Terminal deoxynucleotidyl transferase ‘‘TdT’’
dUTP Nick-End Labeling [TUNEL] method), and malondialdehyde (MDA) formation, respectively. Outcomes of neurocognitive state were operationally defined as Morris water maze test variables «escape latency» and «number of platform crossings». Records were selected based on the following inclusion criteria: institutional access, English language, in-tissue significant measurements in CNS from mice or rats injected with dexmedetomidine i.p., uninfluenced from age extremes. Exclusion criteria included: retracted articles, serum/plasma measurements, mRNA measurements, vague subject description, vehicle different from saline, species different from mice or rats, and studies with long-term experimental design.

9.2.2 Information Sources and Search Strategy

On July 15th 2022, the National Library of Medicine was last consulted through PubMed using the following query strings: “(dexmedetomidine) AND ((IL-1beta) OR (IL-1b))” (194 records from 2007 to 2022), “dexmedetomidine IL-6” (313 records from 2001 to 2022), “dexmedetomidine tunel” (90 records from 2008 to 2022), “dexmedetomidine mda” (124 records from 2004 to 2022), “(dexmedetomidine) AND (escape latency)” (22 records from 2007 to 2022), “(dexmedetomidine) AND (platform) AND ((cross) OR (crossing))” (14 records from 2016 to 2022), “dexmedetomidine morris” (58 records from 2007 to 2022) without any filters or limits.

9.2.3 Selection and Data Collection Processes

Three reviewers (SG, CA, DA) inspected the title, abstract, and/or full text for each record for inclusion criteria and existence of operational outcomes independently and without any automation tool. Discrepancies were sorted out over a discussion round including all authors and led by the 3 reviewers. The same 3 reviewers collected the data from each of the eligible records in an independent fashion without any automation tool.

9.2.4 Data and Synthesis

Data of operationally defined variables (i.e., IL-1β, IL-6, apoptosis rate, MDA formation, escape latency, and number of platform crossings) was extracted by 4 reviewers (SG, CA, YP, DA), tabulated, organized, and complemented with the following auxiliary variables: main article theme, measured tissue, species, animal weight, dexmedetomidine dose and route, and sample size (n). Data expressed as median ± IQR was transformed to mean ± SD using the method by Hozo et al. [33] and error data expressed as SEM was transformed to SD using the formula: SD = SEM * SQRT(n). All variables in the dataset were complete except for animal weight, which was imputed for 7 (out of 115 records) from nomogram charts published elsewhere [34–38]. Because animal weight was not homogeneous, a correction factor for normalizing dexmedetomidine dose was introduced (dose [µg/kg i.p.]/weight [g]) to allow full dataset comparison. Thus, raw input for meta-analyses consisted of normalized mean, normalized SD, and n. Synthesis was performed per outcome by calculating the standardized mean difference (SMD, bias corrected Hedge’s G) between the injury group as control and the injury + dexmedetomidine group as treatment. Because some studies tested more than a single dose [39–48] or more than a single type of tissue [42, 47], the term record was assigned to a single measurement (single dose in a single type of tissue). Thus, the number of records in each outcome dataset was greater than the number of studies. For the synthesis, raw datasets were loaded on R 4.1.1 and a first-round meta-analysis was performed using the General Package for Meta-Analysis (meta) [49]. Then, outliers were queried using the dmetar package [50] and further identified based on p-value and confidence interval.
(CI) overlap, i.e., studies with a *p*-value > 0.05 and a CI not contained within the CI interval of the pooled effect. Subsequently, a second-round meta-analysis was performed with filtered studies, which was then queried for the presence and extent of statistical heterogeneity using the meta package (Tables S7–S12). Lastly, a third-round meta-analysis was performed with filtered records to obtain the final set of results. No subgroup or meta-regression was performed. As part of the synthesis, heterogeneity contribution was visually displayed using Baujat plots (Tables S7–S12) [51], individual and pooled effects were visually displayed using forest plots, and effect symmetry and study precision were visually displayed using contour-enhanced funnel plots (Figs. S1–S6) [52].

### 9.2.5 Risk of Bias and Certainty Assessment

Four reviewers (SG, CA, YP, DA) independently assessed eligible records individually and comprehensively for bias and certainty using the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE)’s risk of bias tool [53] and the Essential 10 set from the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0 [54], without the use of automation. Risk of bias due to missing results was assessed as a part of the SYRCLE’s risk of bias tool.

### 9.3 Results

The search and identification process proceeded by outcome: out of 194 total studies for IL-1\(\beta\), 172 were excluded and 22 were included [24, 39, 40, 47, 55–72] (Fig. 9.1a), out of 313 total studies for IL-6, 300 were excluded and 13 were included [24, 41, 42, 56, 59–62, 65, 67, 73–75] (Fig. 9.1b), out of 90 total studies for apoptosis rate, 71 were excluded and 19 were included [24, 28, 40, 43–47, 58, 59, 70, 72, 73, 76–81] (Fig. 9.1c), out of 124 total studies for MDA formation, 117 were excluded and 7 were included [47, 48, 56, 67, 70, 82, 83] (Fig. 9.1d), out of 80 total studies for escape latency, 73 were excluded and 7 were included [39, 41, 67, 80, 84–86] (Fig. 9.1e), and out of 72 total studies for number of platform crossings, 68 were excluded and 4 were included [39, 67, 85, 86] (Fig. 9.1f). Behavioral outcomes received record input from search string “dexmedetomidine morris”, which yielded 58 studies and contributed with 4 eligible studies for escape latency and 3 eligible studies for platform crossings. Final number of eligible records (as defined in the methods section) was 25 for IL-1\(\beta\) (Table S1), 15 for IL-6 (Table S2), 27 for apoptosis rate (Table S3), 8 for MDA formation (Table S4), 9 for escape latency (Table S5), and 5 for platform crossings (Table S6). Inclusion criteria was restrictive enough to allow for deterministic exclusion.

### 9.3.1 Study Characteristics

Eligible records corresponded to 67 measurements in the brain (14 in the cortex, 33 in the hippocampus, 1 in the striatum, and 19 from unspecified cerebral regions) and 8 in the spinal cord from mice of the BALB/C, C57BL/6, CD-1, ICR, and Swiss albino strains, and from rats of the Sprague Dawley and Wistar strains. Mice weighted an average of 26.9 g and were injected i.p. with dexmedetomidine at doses ranging from 10 to 500 \(\mu\)g/kg. Rats weighted an average of 246 g and were injected i.p. with dexmedetomidine at doses ranging from 1 to 800 \(\mu\)g/kg. Injury models included traumatic brain injury, post-operative cognitive disorder, sepsis, ischemia/reperfusion, among others. A comprehensive list of injury models, animal characteristics, and doses for eligible records is available in Table 9.1.

Interleukin-1\(\beta\) was measured primarily in the brain (23 records against 2 records in the spinal cord) from mice of the BALB/C, C57BL/6, CD-1, ICR, and Swiss albino strains, and from rats of the Sprague Dawley strain. Mice weighted an average of 28.6 g and were injected i.p. with dexmedetomidine at doses ranging from 20 to 500 \(\mu\)g/kg. Rats weighted an average of 229.6 g
and were injected i.p. with dexmedetomidine at doses ranging from 10 to 800 µg/kg. A comprehensive list of injury model, animal characteristics, and doses for eligible records is available in Table S1.

Interleukin-6 was measured primarily in the brain (14 records against 1 record in the spinal cord) from mice of the C57BL/6, CD-1, and ICR strains, and from rats of the Sprague Dawley and Wistar strains. Mice weighted an average of 28.3 g and were injected i.p. with dexmedetomidine at doses ranging from 10 to 150 µg/kg. Rats weighted an average of 236.4 g and were injected i.p. with dexmedetomidine at doses ranging from 4 to 50 µg/kg. A comprehensive list of injury model, animal characteristics, and doses for eligible records is available in Table S2.

Apoptosis rate was measured using TUNEL primarily in the brain (22 records against 5 record in the spinal cord) from mice of the BALB/C and C57BL/6 strains, and from rats of the Sprague Dawley strain. Mice weighted an average of 24.2 g and were injected i.p. with dexmedetomidine at doses ranging from 25 to 500 µg/kg. Rats weighted an average of 262.5 g and were injected i.p. with dexmedetomidine at doses ranging from 1 to 200 µg/kg. A comprehensive list of injury model, animal characteristics, and doses for eligible records is available in Table S3.

Malondialdehyde formation was measured exclusively in the brain from mice of the BALB/C and C57BL/6 strains, and from rats of the Sprague Dawley and Wistar strains. Mice weighted an average of 24.3 g and were injected i.p. with dexmedetomidine at doses ranging from 20 to 25 µg/kg. Rats weighted an average of 240 g and were injected i.p. with dexmedetomidine at doses ranging from 5 to 100 µg/kg. A comprehensive list of injury model, animal characteristics, and doses for eligible records is available in Table S4.

Escape latency was measured in mice of the C57BL/6 and ICR strains, and from rats of the Sprague Dawley strain. Mice weighted an...
Table 9.1  Sequential meta-analysis model refinement for each outcome appraised

| Outcome                          | Analysis          | N   | SMD   | p      | 95% CI          | 95% PI          | $I^2$ (%) | 95% CI    | Q     | p       |
|----------------------------------|-------------------|-----|-------|--------|-----------------|-----------------|-----------|-----------|-------|---------|
| **IL-1β**                        | Raw analysis      | 25  | -4.786| < 0.0001 | [-6.0682; -3.5029] | [-11.0328; 1.4617] | 87.40     | [82.7%; 90.9%] | 190.76 | < 0.0001 |
|                                  | OSP assessment    | 18  | -4.910| < 0.0001 | [-5.7547; -4.0653] | [-7.9134; -1.9065] | 65.90     | [44.0%; 79.3%] | 49.89  | < 0.0001 |
|                                  | Infl. cases excluded | 15  | -4.282| < 0.0001 | [-4.8395; -3.7247] | [-4.8965; -3.6677] | 18.10     | [0.0%; 55.1%]  | 17.1   | 0.2507  |
| **IL-6**                         | Raw analysis      | 15  | -11.36| 0.0005  | [-17.5285; -5.1821] | [-36.8379; 14.1274] | 87.30     | [80.7%; 91.7%] | 110.41 | < 0.0001 |
|                                  | OSP assessment    | 9   | -5.649| < 0.0001 | [-6.6763; -4.6207] | [-8.0694; -3.2276] | 27.10     | [0.0%; 66.0%]  | 10.97  | 0.2034  |
|                                  | Infl. cases excluded | 14  | -5.980| < 0.0001 | [-6.7658; -5.1951] | [-7.6019; -4.3590] | 32.90     | [0.0%; 64.6%]  | 19.37  | 0.112   |
| **TUNEL**                        | Raw analysis      | 27  | -6.521| < 0.0001 | [-8.0214; -5.0204] | [-14.1191; 1.0773] | 87.60     | [83.1%; 90.9%] | 209.18 | < 0.0001 |
|                                  | OSP assessment    | 18  | -6.795| < 0.0001 | [-7.9142; -5.6758] | [-10.9903; -2.5997] | 67.30     | [46.5%; 80.0%] | 52     | < 0.0001 |
|                                  | Infl. cases excluded | 14  | -5.980| < 0.0001 | [-6.7658; -5.1951] | [-7.6019; -4.3590] | 32.90     | [0.0%; 64.6%]  | 19.37  | 0.112   |
| **MDA**                          | Raw analysis      | 8   | -4.759| 0.0002  | [-7.2585; -2.2585] | [-13.8356; 4.3186] | 93.80     | [90.0%; 96.2%] | 113.01 | < 0.0001 |
|                                  | OSP assessment    | 7   | -4.114| 0.0012  | [-6.6033; -1.6255] | [-13.0236; 4.7948] | 90.40     | [82.7%; 94.6%] | 62.24  | < 0.0001 |
|                                  | Infl. cases excluded | 5   | -1.918| < 0.0001 | [-2.4342; -1.4011] | [-2.7564; -1.0789] | 43.40     | [0.0%; 79.2%]  | 7.07   | 0.1322  |
| **Escape latency**               | Raw analysis      | 9   | -12.50| 0.1291  | [-28.6375; 3.6440] | [-73.7227; 48.7292] | 95.10     | [92.5%; 96.7%] | 161.66 | < 0.0001 |
|                                  | OSP assessment    | 7   | -4.258| 0.0007  | [-6.7092; -1.8064] | [-12.8636; 4.3749] | 89.30     | [80.5%; 94.2%] | 56.26  | < 0.0001 |
|                                  | Infl. cases excluded | 3   | -2.428| < 0.0001 | [-3.2641; -1.5925] | [-7.8467; 2.9901] | 0         | [0.0%; 89.6%]  | 0.32   | 0.8519  |
| **Platform crossings**           | Raw analysis      | 5   | 6.880 | 0.0008  | [2.8570; 10.9038] | [-8.1549; 21.9157] | 96.40     | [93.8%; 97.9%] | 109.73 | < 0.0001 |
|                                  | OSP assessment    | 4   | 9.138 | < 0.0001 | [6.8230; 11.4531] | [0.6758; 17.6003] | 45.50     | [0.0%; 81.9%]  | 5.5    | 0.1383  |

SMD Standardized mean difference, CI Confidence interval, PI Predictive interval, $I^2$ Higgins and Thompson’s $I^2$ Statistic, Q Cochran’s Q, OSP Outlier, significance, and precision assessment
average of 27.5 g and were injected i.p. with dexmedetomidine at doses ranging from 10 to 20 µg/kg. Rats weighted an average of 248.3 g and were injected i.p. with dexmedetomidine at doses ranging from 15 to 40 µg/kg. A comprehensive list of injury model, animal characteristics, and doses for eligible records is available in Table S5.

Number of platform crossings was measured in mice of the C57BL/6 strain and from rats of the Sprague Dawley strain. Mice weighted an average of 22.5 g and were injected i.p. with dexmedetomidine at a dose of 20 µg/kg. Rats weighted an average of 230 g and were injected i.p. with dexmedetomidine at doses ranging from 20 to 40 µg/kg. A comprehensive list of injury model, animal characteristics, and doses for eligible records is available in Table S6.

9.3.2 Results of Syntheses

Raw meta-analysis of in-tissue levels of IL-1β suggested a significantly protective effect for dexmedetomidine against insult (SMD = −4.786), but with a significant level of heterogeneity between studies (I² = 87.4%) (Table 9.1). Outlier, significance, and precision (OSP) assessment identified 7 studies distorting the random-effects model [47, 57, 60, 63, 68]. A second-round meta-analysis, after OSP exclusion, suggested a significantly protective effect for dexmedetomidine against insult (SMD = −5.6485), with no record significantly contributing to model heterogeneity (I² = 27.1%, Table 9.1, Table S8). Visual inspection of effect symmetry and study precision (risk of bias among contributing studies) showed that second-round records fell within the area of 95% confidence of the overall effect in a symmetrical fashion (Fig. 9.2), corroborating precision in exclusion criteria (Table S8) and meta-analysis refinement (Table 9.1). This latter round was considered final and was visually characterized in detail using a forest plot (Fig. 9.2).

Raw meta-analysis of in-tissue levels of IL-6 suggested a significantly protective effect for dexmedetomidine against insult (SMD = −11.3553), but with a significant level of heterogeneity between studies (I² = 87.30%). Outlier, significance, and precision assessment identified 6 records distorting the random-effects model [41, 42, 60, 73]. A second-round meta-analysis, after OSP exclusion, revealed a significantly protective effect for dexmedetomidine against insult (SMD = −5.6485), with no record significantly contributing to model heterogeneity (I² = 27.1%, Table 9.1, Table S8). Visual inspection of effect symmetry and study precision (risk of bias among contributing studies) showed that second-round records fell within the area of 95% confidence of the overall effect in a symmetrical fashion (Fig. S2), corroborating precision in exclusion criteria (Table S8) and meta-analysis refinement (Table 9.1). This latter round was considered final and was visually characterized in detail using a forest plot (Fig. 9.3).

Raw meta-analysis of apoptosis rate (measured using TUNEL) suggested a significantly protective effect for dexmedetomidine against insult (SMD = −6.5209), but with a significant level of heterogeneity between studies (I² = 87.6%). Outlier, significance, and precision (OSP) assessment identified 9 records distorting the random-effects model [40, 43, 47, 59, 79, 81]. A second-round meta-analysis, after OSP exclusion, suggested a significantly protective effect for dexmedetomidine against insult (SMD = −6.795), but with a significant level of heterogeneity between studies (I² = 67.3%). Heterogeneity assessment further identified 4 records considerably contributing to overall heterogeneity [40, 73, 77, 80] (Table S9).

A third-round meta-analysis, excluding cases with evident influence over model heterogeneity, revealed a significantly protective effect for dexmedetomidine against insult (SMD = −5.9804) with a significant level of homogeneity
between studies ($I^2 = 32.9\%$) (Table 9.1). Visual inspection of effect symmetry and study precision (risk of bias among contributing studies) showed that third-round records fell within the area of 95% confidence of the overall effect in a symmetrical fashion (Fig. S3), except for 1 record [44], which was attributed the smallest weight in the model. Thus, round iteration resulted in a gain in precision in exclusion criteria (Table S9) and meta-analysis refinement (Table 9.1). This latter round was considered final and was visually characterized in detail using a forest plot (Fig. 9.4).

Raw meta-analysis of in-tissue formation of MDA suggested a significantly protective effect for dexmedetomidine against insult (SMD = −4.7585), but with a significant level of heterogeneity between studies ($I^2 = 93.80\%$). Outlier, significance, and precision (OSP) assessment identified 1 study distorting the random-effects model [67]. A second-round meta-analysis, after OSP exclusion, suggested a significantly protective effect for dexmedetomidine against insult (SMD = −4.1144), but with a significant level of heterogeneity between studies ($I^2 = 90.40\%$). Heterogeneity assessment

| Record [Citation] | SMD  | 95% CI    | p-value  | Weight |
|-------------------|------|-----------|----------|--------|
| Wang Z et al. (2018) [64] | −4.24 | [−5.46; −3.01] | < 0.01  | 20.8%  |
| Zhang Y et al. (2021) [40] | −3.01 | [−4.56; −1.47] | < 0.01  | 13.0%  |
| Zhang Y et al. (2021) [40] | −3.43 | [−5.10; −1.75] | < 0.01  | 11.1%  |
| Xu KL et al. (2018) [55] | −4.17 | [−5.85; −2.49] | < 0.01  | 11.0%  |
| Li R et al. (2020) [70] | −3.87 | [−5.68; −2.05] | < 0.01  | 9.4%   |
| Wang YL et al. (2021) [61] | −4.22 | [−6.16; −2.29] | < 0.01  | 8.3%   |
| Hu J et al. (2018) [56] | −4.43 | [−6.44; −2.42] | < 0.01  | 7.7%   |
| Chen L et al. (2019) [62] | −4.63 | [−7.13; −2.13] | < 0.01  | 5.0%   |
| Lu Y et al. (2017) [57] | −3.88 | [−6.82; −0.94] | < 0.01  | 3.6%   |
| Wang D et al. (2018) [69] | −6.68 | [−10.58; −2.78] | < 0.01  | 2.0%   |
| Li F et al. (2019) [59] | −8.07 | [−12.12; −4.02] | < 0.01  | 1.9%   |
| Zhang L et al. (2021) [63] | −5.79 | [−9.90; −1.68] | < 0.01  | 1.8%   |
| Li P et al. (2021) [40] | −7.49 | [−11.81; −3.17] | < 0.01  | 1.7%   |
| Karakaya F et al. (2022) [72] | −7.79 | [−12.27; −3.31] | < 0.01  | 1.5%   |
| Zhu YJ et al. (2016) [71] | −9.08 | [−14.26; −3.91] | < 0.01  | 1.2%   |

Random effects model $-4.28$ [ $-4.84; -3.72$] $100.0\%$

Heterogeneity: $I^2 = 18\%$, $t^2 < 0.0001$, $p = 0.25$

Fig. 9.2 Forest plot depicting pooled effect of dexmedetomidine on levels of IL-1β in animal CNS after insult

| Record [Citation] | SMD  | 95% CI    | p-value  | Weight |
|-------------------|------|-----------|----------|--------|
| Guo B et al. (2021) [75] | −6.91 | [−8.28; −5.54] | < 0.01  | 21.8%  |
| Hu J et al. (2018) [60] | −4.48 | [−6.51; −2.46] | < 0.01  | 14.9%  |
| Tian M et al. (2021) [24] | −3.51 | [−5.55; −1.48] | < 0.01  | 14.9%  |
| Minaei A et al. (2019) [59] | −5.16 | [−7.42; −2.89] | < 0.01  | 13.1%  |
| Li R et al. (2020) [67] | −5.50 | [−7.89; −3.11] | < 0.01  | 12.2%  |
| Feng X et al. (2021) [61] | −6.06 | [−9.63; −2.48] | < 0.01  | 6.7%   |
| Li P et al. (2019) [65] | −7.53 | [−11.32; −3.73] | < 0.01  | 6.1%   |
| Wang D et al. (2018) [62] | −6.92 | [−10.94; −2.90] | < 0.01  | 5.5%   |
| Zhang X et al. (2018) [73] | −7.44 | [−11.74; −3.14] | < 0.01  | 4.9%   |

Random effects model $-5.65$ [ $-6.68; -4.62$] $100.0\%$

Heterogeneity: $I^2 = 27\%$, $t^2 = 0.7732$, $p = 0.20$

Fig. 9.3 Forest plot depicting pooled effect of dexmedetomidine on levels of IL-6 in animal CNS after insult
further identified 2 studies considerably contributing to overall heterogeneity [56, 82] (Table S10). A third-round meta-analysis excluding cases with evident influence over model heterogeneity revealed a significantly protective effect for dexmedetomidine against insult (SMD = −1.9176) with a significant level of homogeneity between studies (I² = 43.4%) (Table 9.1). Visual inspection of effect symmetry and study precision (risk of bias among contributing studies) showed that third-round records fell within the area of 95% confidence of the overall effect in a symmetrical fashion (Fig. S4), except for 1 study [70], which was attributed the smallest weight in the model. Thus, round iteration resulted in a gain in precision in exclusion criteria (Table S10) and meta-analysis refinement (Table 9.1). This latter round was considered final and was visually characterized in detail using a forest plot (Fig. 9.5).

Raw meta-analysis of behavioral outcome escape latency suggested a significantly protective effect for dexmedetomidine against insult (SMD = −12.4967), but with a significant level of heterogeneity between studies (I² = 95.1%). Outlier, significance, and precision assessment identified 2 records distorting the random-effects model [67, 86]. A second-round meta-analysis, after OSP exclusion, revealed a significantly protective effect for dexmedetomidine against insult (SMD = −4.2578), but with a significant level of heterogeneity between studies (I² = 89.3%). Heterogeneity assessment further identified 4 records considerably contributing to overall heterogeneity [41, 80, 85] (Table S11). A third-round meta-analysis, excluding cases with evident influence over model heterogeneity, revealed a significantly protective effect for dexmedetomidine against insult (SMD = −2.4283) with an abrogated heterogeneity between studies (I² = 0%) (Table 9.1). Visual inspection of effect symmetry and study precision (risk of bias among contributing studies) showed that third-round records fell within the area of 95% confidence of the overall effect in a symmetrical fashion (Fig. S5), corroborating precision in exclusion criteria (Table S11) and meta-analysis refinement (Table 9.1). This latter round was considered final and was visually characterized in detail using a forest plot (Fig. 9.6).

Raw meta-analysis of behavioral outcome platform crossings suggested a significantly protective effect for dexmedetomidine against insult (SMD = 6.8804), but with a significant level of heterogeneity between studies (I² = 96.4%). Outlier, significance, and precision
assessment identified 1 record distorting the random-effects model [67, 86]. A second-round meta-analysis, after OSP exclusion, revealed a significantly protective effect for dexmedetomidine against insult (SMD = 9.1381), with no record significantly contributing to model heterogeneity ($I^2 = 45.5\%$, Table 9.1, Table S12). Visual inspection of effect symmetry and study precision (risk of bias among contributing studies) showed that second-round records fell within the area of 95% confidence of the overall effect in a symmetrical fashion (Fig. S6), corroborating precision in exclusion criteria (Table S12) and meta-analysis refinement (Table 9.1). This latter round was considered final and was visually characterized in detail using a forest plot (Fig. 9.7).

### 9.3.3 Risk of Bias and Certainty of Evidence

Risk of bias measured using the SYRCLE’s tool was low for all eligible studies in all domains except for «Allocation concealment», «Random housing», «Random outcome assessment», and «Blinding» (Fig. S7), for which an unclear risk of bias was found in all eligible studies. No eligible studies with high risk of bias were found. No risk of bias due to missing results were found. Certainty of evidence measured using the ARRIVE Guidelines 2.0 found transparent and accurate reporting in every domain except for Item 2b «How sample size was decided», Items 3a and 3b «Criteria used for including and excluding animals», Item 4a «Criteria for randomization», Item 4b «Control for confounders, Item 5 «Blinding», for all eligible studies, and Item 8b «Provide further relevant information on the provenance of animals» for only 8 studies [41, 43, 46, 47, 57, 77, 79, 81] (Table S14).

### 9.4 Discussion

Dexmedetomidine is almost univocally considered neuroprotective. Although efforts to establish its value as such have been made in the form...
of review articles [87], a literature gap in this regard is widely recognized. To bridge this literature gap in an objective manner, the latest and most elaborate endeavor, a systematic review [88], found dexmedetomidine effects to be heterogenous and occasionally counter-intuitive. The present study recognizes the lack of a robust and definitive appraisal and provides a cohesive assessment for a definitive settlement on the neuroprotective effects of dexmedetomidine.

Stringent appraisal of the neuroprotective potential of dexmedetomidine involved excluding the measurement of outcomes of inflammation, apoptosis, and oxidative stress outside of the CNS. Because of the complex environment and physiological compensations that in vitro settings are unable to replicate, only studies performed in vivo were considered. Among these, the i.v. route of administration was present, but given its dominance among eligible studies and its comparative simplicity, only articles using i.p. delivery were included. To ensure inter-study comparability, and because some physiological and biochemical parameters are not present stably throughout the lifespan of an individual [37], age extremes (i.e., neonatal, postnatal, and aged) were also considered exclusion criteria.

Exploration of sources of potential heterogeneity showed that the analysis could not have been carried out without introducing an arithmetic association between animal dose and weight. Also, the process of meta-analysis refinement did not exhibit a preference for a type of species except for TUNEL, for which most of the records ruled out corresponded to data from rats (Table S13). However, for this case, most of the records included in the analysis corresponded to measurements in rats, so species representativity was ultimately balanced. Additionally, the process of meta-analysis refinement did not exhibit a preference for type of structure within the tissue, as the number of structures in each group resulted relatively even, except for MDA formation, for which the number of records measuring the brain (as a whole) was greater in the group of records included in the model (Table S13). The physiological relevance of this instance may be interesting to explore empirically. Furthermore, and regarding only records that were included in the final model, a general parity was evidenced between species, except for behavioral variables, for which all results in mice were ruled out (Table S13). The reason for this blunt type of exclusion is unexpected and may represent an area of interest for further research.

Although a protective effect of dexmedetomidine on inflammatory cytokines was indeed expected, the contrastive pooled effect between IL-1β (pooled SMD = −4.84, Table 9.1) and IL-6 (pooled SMD = −5.65) may be a corroboration [60] of the signaling preference through α2AAR over α2BAR. Besides canonical pathways converging in NF-κB phosphorylation [89], dexmedetomidine has been described to down-regulate pro-inflammatory cytokine synthesis and release through the PI3K/Akt/mTOR [80], NLRP3/caspase1 [39], and Keap1/Nrf2 [90] pathways. Although Nrf2 may engage additional transcription factors, including NF-kB [91, 92], it is essential in redox homeostasis and responses to reactive oxygen species [93, 94]. In fact, Nrf2

### Table 9.1

| Record [Citation] | SMD | 95% CI   | p-value | SMD | Weight |
|------------------|-----|----------|---------|-----|--------|
| Guo B et al. (2021) [67] | 11.00 | [8.90; 13.09] | < 0.01 | | |
| Zhang Y et al. (2021) [39] | 7.28 | [4.24; 10.33] | < 0.01 | | |
| Zhang Y et al. (2021) [39] | 10.17 | [6.03; 14.31] | < 0.01 | | |
| Deng F et al. (2020) [86] | 6.03 | [0.33; 11.74] | 0.04 | | |

Random effects model 9.14 [6.82; 11.45]

Heterogeneity: $I^2 = 46\%$, $T^2 = 2.4729$, $p = 0.14$

![Forest plot depicting pooled effect of dexmedetomidine on behavioral variable «platform crossings» in animal CNS after insult](image-url)
has been reported to mediate neuroprotection through the Nrf2/HO-1/NAD(P)H/NQO1 pathway [95] and by limiting mitochondrial ROS production [23]. However, the considerably lower magnitude of protection against MDA formation (pooled SMD = −1.92) and the smaller number of records included in the final-round meta-analysis may represent a mechanistic suggestion about how distant α2AAR (and α2BAR) and Nrf2 signaling pathways are. Thus, further experimental characterization may contribute to the understanding of the low performance of dexmedetomidine over MDA formation that the present study found (pooled SMD = −1.9). Nonetheless, Nrf2 has also been found to mediate dexmedetomidine action against apoptosis [95]. As expected, because dexmedetomidine signaling through JAK2/STAT3 [96, 97] and PI3K/AKT/GSK3β [98, 99] has been previously documented, a protective effect against apoptosis (pooled SMD = −5.98) was found. In sum, by selectively appraising its effects in the CNS—at the cellular/tissular level, dexmedetomidine alone is cohesively able to protect from inflammation and apoptosis, and less cohesively able to protect from oxidative stress. Thus, if neuroprotection is the goal, then the therapeutic potential of dexmedetomidine as an anti-inflammatory and anti-apoptotic agent may be complemented with an antioxidant agent to achieve a comprehensive neuroprotective effect. Although this compound strategy has been explored previously (dexmedetomidine and lidocaine [100], dexmedetomidine and phosphocreatine [101]), finding which agent to pair and at which proportion is a technical challenge with an enormous potential that requires further research.

Behavioral variables (escape latency and number of platform crossings) are de facto a standard for testing hippocampal function [102]. While pooled effect for the former had a comparatively lower magnitude (pooled SMD = −2.43), pooled effect for the latter elicited the highest magnitude of the present study (Table S13). With the effect of dexmedetomidine over pro-inflammatory cytokine production and apoptosis tested primarily in the hippocampus (Figs. 9.2, 9.3, 9.4 and 9.5 could, at least, partially explain why and how animals in the treated group largely overperform their control counterparts, independent from the nature of the injurious agent or physiological insult. This effect, although delineated previously [86, 103, 104], has never been quantified in this manner and represents one of the cornerstones of neuroprotection.

Physiologically, all the proposed intracellular signaling mechanisms by which dexmedetomidine induces its neuroprotective effects are associated to synaptic plasticity and have a prominent role in brain pathology. For example, hippocampal Rac1 is associated to memory consolidation, maintenance, and memory loss [105–109], and its inhibition increases memory and synaptic plasticity [110]. The JNK pathway regulates memory formation, synaptic plasticity and is an important target for neurodegenerative diseases [111]. The NR2B/ERK pathway includes the activation of NMDA-type glutamatergic receptors (NMDAR) and the subsequent activation of the ERK signaling pathway, which are critical for synaptic plasticity and memory [112, 113] and are associated to neurodegenerative diseases [114–116]. Lastly, the neurotrophic factor BDNF, through its different signaling pathways, and specially through TrkB-dependent signaling, has an important role in regulating synaptic plasticity and dendritic growth, and is associated to cognitive deficits in neurodegenerative disorders [117].

Since the proposed mechanisms for hippocampal protection include several signaling pathways that are critical for hippocampal function and are affected by neurodegenerative disorders and insults, including the Rac1/AKT/NF-κB [67], the JNK pathway [118], NR2B/ERK [119], and BDNF-TrkB-CREB [120] signaling pathways, therapeutic explorations with dexmedetomidine and other antioxidant agents that target these signaling cascades in the hippocampus may represent the ultimate neuroprotective formula against a myriad of physiological insults.
9.5 Strengths and Limitations

Foremost, the ability to size events occurring at the molecular/cellular level and their translation into behavior and cognition at the same time is one of the key strengths of the present meta-analysis. Then, stringent exclusion criteria constitute an integral feature that added objectivity to our conclusions. Limiting our analysis to measures in the CNS is also a key feature that selectively implicates the action of dexmedetomidine through \( \alpha_{2A} \)AR and \( \alpha_{2B} \)AR. Interestingly, segmentation of analysis by species did not result in a change in the direction of the effects (data not shown), thus reinforcing the level of falsifiability of the hypothesis on dexmedetomidine regardless of the animal model used to test it (or when individual studies were published, since our meta-analysis did not discriminate by publishing date). Although use of SYRCLE’s risk of bias tool signaled some domains as unclear, because of the biological nature of the outcomes, these could have influenced the readouts in no capacity, and simply represent an excessive sensitivity of the tool for our particular case. Overall risk of bias was low and not a single study was found at high risk (of bias), which reinforces their inclusion. Even though the objective of the ARRIVE Guidelines 2.0 is to maximize transparency and accuracy to ensure reproducibility, an excessive sensitivity was found for Items 2b, 3a, 3b, 4a, 4b, and 5, for, even without thorough details on such items, outcomes for the particular case of the driving question behind this meta-analysis remain inextricable. Overall, key data on sample sizes, subject identifiers (i.e., species, strain, age, and weight), interventions (i.e., dose, route of administration, and type of vehicle), and statistical analyses to gauge outcomes, were deemed satisfactory enough to ensure reproducibility, which justifies their inclusion. From a methodological perspective, several decisions, based on thorough scientific criteria, that had to be taken to carry out the analyses could be pointed as possible sources of error, e.g., using the average weight of animals in each record, using published data to impute animal weight from age, and using TUNEL as the only measure of apoptosis. Nevertheless, we estimate the actual contribution to error to be marginal as demonstrated by the level of significance in third-round meta-analyses and the degree of symmetry in funnel plots (Figs. S1–S6).

9.6 Conclusions

The present meta-analysis corroborates the neuroprotective action of dexmedetomidine by quantifying its effect on inflammation, apoptosis, oxidative stress, and behavioral variables. Because of its methodological design and previous data on receptor distribution, these effects are suggested to proceed through signaling pathways dependent mainly on \( \alpha_{2A} \)AR in cells of the CNS. Since oxidative stress showed the lowest association with protective effect, it is suggested that intracellular signaling of dexmedetomidine in the CNS may be more dissociated from mechanisms regulating oxidative damage than from inflammation and apoptosis. This study highlights the level complexity that selective tissular interaction with \( \alpha_{2A} \)AR agonist entails at the in vivo level.

Statements and Declarations

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Author Contribution SG, CA, YP, and DA extracted and analyzed the data. SG, CE, CR, JFS, and FS, collectively contributed to manuscript drafting. All authors read and approved the final manuscript.

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Bile Acids Alter the Autophagy and Mitogenesis in Skeletal Muscle Cells

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Abstract

Muscle atrophy decreases muscle mass with the subsequent loss of muscle function. Among the mechanisms that trigger sarcopenia is mitochondrial dysfunction. Mitochondria, whose primary function is to produce ATP, are dynamic organelles that present the process of formation (mitogenesis) and elimination (mitophagy). Failure of any of these processes contributes to mitochondrial malfunction. Mitogenesis is mainly controlled by Peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), a transcriptional coactivator that regulates the expression of TFAM, which participates in mitogenesis. Mitophagy is a process of selective autophagy. Autophagy corresponds to a degradative pathway of protein complexes and organelles. Liver disease caused sarcopenia and increased bile acids in the blood. We demonstrated that the treatment with cholic (CA) or deoxycholic (DCA) bile acids generates mitochondrial dysfunction and loss of biomass. This work assessed whether CA and DCA alter autophagy and mitogenesis. For this, western blot evaluated the autophagy process by determining the protein levels of the LC3II/LC3I ratio. In addition, we assessed mitogenesis using a luciferase-coupled plasmid reporter for the PGC-1α promoter and the protein levels of TFAM by western blot. Our results indicate that treatment with CA or DCA induces autophagy, represented by an increase in the LC3II/LC3I ratio. In addition, a decreased autophagic flux was observed. On the other hand, when treated with CA or DCA, a decrease in the activity of the PGC-1α promoter was observed. However, the levels of TFAM increased in myotubes incubated...
with CA and DCA. Our results demonstrate that CA and DCA modulate autophagy ad mitogenesis in C2C12 myotubes.

Keywords
Mitophagy · Bile acids · Mitochondrial biogenesis · Autophagy · Sarcopenia

Abbreviations
BA Bile acids
CA Cholic acid
CDCA Chenodeoxycholic acid
CCLD Chronic cholestatic liver disease
CREB CAMP response element-binding protein
DCA Deoxycholic acid
ETC Electron transporting chain
FXR Farnesoid receptor
HIF Hypoxia-inducible factor
LCA Lithocholic acid
MHC Myosin heavy chain
NRF Nuclear respiratory transcription factors
OXPHOS Oxidative phosphorylation
PGC-1α Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha
ROS Reactive oxygen species
TDCA Taurodeoxycholic acid
TFAM Transcription Factor A Mitochondrial
TGR5 Takeda-G-protein-receptor-5
UPS Ubiquitin-proteasome system

10.1 Introduction
Skeletal muscle corresponds to most of the body mass, reaching 41.3% of body weight in men and 33.1% in women [1]. Skeletal muscles can convert chemical energy into mechanical energy to generate the force and power that allow us to maintain posture and produce movements. Defining its function from a metabolic perspective, it regulates glycemia and stores essential substrates such as carbohydrates and amino acids [2]. This last has been described as necessary for synthesizing proteins in many tissues, such as the skin, brain, and heart, and maintaining the temperature [3]. In addition, releasing amino acids from skeletal muscle contributes to glycemic control during periods of starvation [2]. Therefore, avoiding any pathological condition affecting muscle mass is critical because it leads to complications associated with movement, muscle strength, and metabolism [2]. Another fundamental characteristic of skeletal muscle is its plasticity, meaning the ability to either increase or decrease its mass, known as hypertrophy and atrophy [4].

Several causes can generate the loss of muscle mass and weakness, including disuse [5], sepsis [6], denervation [7], aging (called primary sarcopenia), and induced by chronic pathologies (secondary sarcopenia). Among chronic diseases that generate sarcopenia are chronic obstructive pulmonary disease, heart failure, and liver disease [8].

Sarcopenia characterizes by a decline in muscle mass and strength and decreased physical activity [9]. Indeed, sarcopenic muscle presents decreased levels of myofibrillar proteins, such as myosin heavy chain (MHC), myosin light chain (MLC), and troponin. These proteins, together with other structural ones, compose the sarcomere, the contractile muscle unit [10, 11]. The mechanism involved in decreased myofibrillar protein levels is an imbalance in the synthesis and degradation protein pathways, highlighting the ubiquitin-proteasome system (UPS) and calpains as the main degradative pathways involved [12].

Another process involved in sarcopenia is autophagy, which can be altered in sarcopenic muscle [13]. Autophagy is responsible for removing damaged cellular components, such as proteins and even organelles [14]. Autophagy requires forming an isolated membrane portion and the post-translational modification of the cytosolic protein LC3I, which conjugates with phosphatidylethanolamine to form LC3II. Then, LC3II incorporates into the isolated membrane, forming the phagophore, capable of engulfing the molecules target that will be charged, creating a
double-membrane vesicle called an autophagosome. This new structure subsequently fusions with the lysosome to form the autolysosome, a compartment where the content inside will be degraded [15].

Finally, the mitochondria are also involved in generating and maintaining sarcopenia. The primary function of mitochondria is to produce ATP. Still, it is also involved in other processes such as apoptosis, autophagy, calcium signaling, and reactive oxygen species (ROS) production [16]. The oxidative phosphorylation (OXPHOS) is carried out by I, II, and III complexes, which constitute the electron transport chain (ETC), together with the ATP synthase. ETC produces not only energy but also ROS. The main ROS formed is superoxide ion (O$_2^-$), which is converted for the cell to hydrogen peroxide (H$_2$O$_2$), a less toxic by-product [17]. However, when there is increased O$_2^-$ and H$_2$O$_2$ levels, they will be converted to hydroxyl ions (OH). These reactive species can oxidize biomolecules, including the ETC component and mitochondrial DNA, resulting in a mitochondrial malfunction that subsequently increases ROS, enhancing the damage [17]. Therefore, normal mitochondrial function requires defense mechanisms, such as antioxidant molecules and enzymes, a system responsible for eliminating misfolded or aggregated proteins, and the mitochondrial life cycle [18]. It consists of a constant mitochondrial remodeling through ongoing events of fission and fusion, a process known as mitochondrial dynamic. On a hand, mitochondrial fusion is mainly modulated by proteins such as Mitofusin 1 and 2 (Mfn 1 and 2) and OPtic Atrophy 1 (Opa 1) proteins [19, 20]. On the other hand, mitochondrial fission is mainly regulated by the proteins Dynamin-Related Protein 1 (Drp1) [19, 20], mitochondrial fission 1 protein (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamic protein 49 and 51 (MiD49, and MiD51), which participate actively in the fragmentation of mitochondria [21].

Several studies have determined the role of mitochondrial fusion in maintaining skeletal muscle function, as also mitochondrial integrity and activity. Thus, a study using muscle-specific KO mice for both Mfn-1 and Mfn-2 shows that mice develop mitochondrial dysfunction, accumulation of mitochondrial DNA damage, and deficit in growth [22]. In comparison, a KO mouse for Mfn2 in skeletal muscle was shown to result in oxidative stress and sarcopenia in adult mice [23]. Interestingly, the deletion of Mfn1 and Mfn2 in adult skeletal muscle resulted in a decline in muscle function, evidenced by a significant decrease in exercise performance, indicating that mitochondrial fusion is essential to physical activity in mice [24]. Another antecedent shows that specific deletion of Opa1 in skeletal muscle produces not only mitochondrial dysfunction but also oxidative stress, endoplasmic reticulum (ER) stress, and inflammation [25–27]. In addition, Opa1 deficiency also induces the secretion of Fibroblast Growth Factor 21 (FGF21) from skeletal muscle, mechanistically explaining some alterations such as lipid dysregulation, inflammation, and the senescence of different tissues [25]. These antecedents indicate that mitochondrial fusion proteins are essential for maintaining mitochondrial and skeletal muscle function.

Regarding the fission process, some antecedents indicate its role in maintaining skeletal muscle health. The genetic silencing of Fis1 and Drp1 in skeletal muscle and the consequent inhibition of mitochondrial fission has been shown to prevent muscle wasting induced by two models of muscle atrophy, such as starvation or overexpression of FoxO3a [28]. Muscle-specific Drp1 KO mice show a severe myopathic phenotype, including muscle wasting, weakness, and evidence of muscle degeneration and regeneration [29]. Another study with muscle-specific Drp1 knockdown showed a decrease in mitochondrial respiration and increased muscle regeneration, denervation, fibrosis, and oxidative stress [30]. Interestingly, both Drp1 KO and knockdown presented altered autophagy and mitophagy [29, 30]. These mice also showed muscle atrophy [29, 30], indicating that Drp1 is crucial in regulating skeletal muscle development and maintenance. The antecedent reinforces this observation showing that muscle-specific Drp1 overexpression altered skeletal muscle growth in mice [31, 32]. Another study showed that loss of
Fis1 leads to mitochondrial dysfunction, proteostasis impairment, and muscle degeneration in Drosophila [33]. Taken altogether, the antecedents presented above clearly indicate that mitochondrial fission is essential for skeletal muscle function.

Other events that belong to the mitochondrial life cycle are the production of new mitochondria, known as mitochondrial biogenesis or mitogenesis, and the elimination of damaged mitochondria through mitophagy, a process of selective autophagy for mitochondria [34]. These processes are connected, and any alteration in them leads to mitochondrial dysfunction, characterized by low ATP production and/or increased ROS [35].

Mitogenesis is defined as the growth of pre-existing mitochondria and their eventual division, increasing the number, size, and mass of mitochondria [36, 37]. This process is induced by exercise, caloric restriction, low temperature, oxidative stress, cell division, and differentiation [36, 37]. Mitochondrial proteins are synthesized in two genomes. The mitochondrial genome contains 13 genes (37 total) that codify for components participating in OXPHOS [36, 37]. In addition, the nuclear genome encodes 1000–1500 proteins, which are synthesized on cytosolic ribosomes and imported to mitochondria [38]. Therefore, biogenesis requires the coordination of the two genomes involved. The master regulator of mitogenesis is the protein peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha (PGC-1α). This protein is a cytosolic transcriptional co-activator that, in response to biogenesis-inducing stimuli, undergoes post-translational modifications to translocate to the nucleus, interact with different transcription factors, and regulate the target-gene transcription [36, 37]. Among these transcriptional factors are myocyte-enhancing factor-2 (MEF2) and nuclear respiratory transcription factors 1 and 2 (NRF-1 and NRF-2) [39]. NRF-1 induces the expression of mitochondrial transcription factor A (TFAM), which, once synthesized, translocates to the mitochondria to induce the expression of mitochondrial genes, such as components of the ETC, together to regulate mitochondrial DNA replication [40]. NRF-2 induces the expression of various genes, including NAD(P)H quinone oxidoreductase 1, glutathione S-transferase, and subunit 4 of the hetero-oligomeric enzyme cytochrome c oxidase (COX IV) [41].

The disruption of mitochondrial biogenesis has been described in sarcopenia. In a murine model of colorectal cancer-induced sarcopenia, decreased PGC-1α protein levels and target genes for PGC-1α, such as cytochrome C and COX IV, were found [42]. In other studies, it was described that the decrease in mitogenesis precedes muscle weakness. Therefore, mitochondrial biogenesis is a cause of sarcopenia and not a consequence [42–44]. It has been described a decrease in PGC-1α in sarcopenic conditions such as obesity and type 2 diabetes in mouse and human muscles [45, 46]. Therefore, alterations in mitogenesis are closely linked to the development of sarcopenia.

Diverse soluble factors generate sarcopenia, among them bile acids [47]. Bile acids are amphipathic molecules produced in the liver from cholesterol and stored in the gallbladder [48]. They divide according to their origin into primary BA, the most abundant and synthesized in the liver, such as cholic acid (CA) and chenodeoxylic acid (CDCA), or secondary BA (or dehydroxylated derivates), which are primary BA modified by intestinal bacteria. These are the acids deoxycholic (DCA) and lithocholic (LCA) [48, 49]. The most abundant BA are CA, CDCA, and DCA [50]. Bile acids are synthesized in hepatocytes from cholesterol [51]. Once synthesized, they are secreted from the hepatocyte to the bile duct and discharged into the small intestine [52]. About 95% of bile acids are recycled back to the liver through enterohepatic circulation [52].

Bile acids also are essential in pathologies such as cholestatic chronic liver diseases (CCLD). A typical characteristic of CCLD is the increased serum levels of bile acids [53, 54]. CCLD is a pathology characterized by progressive liver damage, eventually leading to cirrhosis. This last stage presents different alterations, such as liver fibrosis and hepatocellular dysfunction.
Therefore, it ultimately leads to a liver transplant. Of patients with cirrhosis, 40–80% have muscle weakness and develop sarcopenia, the loss of muscle mass, strength, and function [56, 57]. This condition has profound implications for the transplant since people who present both sarcopenia and cirrhosis have lower post-transplant survival (40%), in contrast to people who do not show muscle weakness (80%) [58]. Besides, sarcopenia could help predict mortality in cirrhotic patients [59]. In these pathologies, a decrease in muscle mass has been described in animal models and humans [58, 60].

Bile acids have gastrointestinal functions widely described, such as lipid digestion, dietary protein denaturation, and antimicrobial effects [51]. However, recent studies have indicated bile acids as signaling molecules through two receptors, Farnesoid X (FXR) and TGR5 [61, 62]. FXR is a nuclear receptor in the liver, adrenal glands, kidney, and intestinal tract [63]. TGR5 is a G protein-coupled membrane receptor that increases energy expenditure when activated in brown adipose tissue [62, 64]. In addition, TGR5 has been described as an essential regulator of blood glucose since promoting the release of insulin in the pancreatic β cells [65, 66]. In non-ciliated cholangiocytes, TGR5 induces proliferation. However, in ciliated cholangiocytes, proliferation is inhibited [67]. Therefore, it is possible to observe opposite effects in a cell-dependent manner. The metabolic role of TGR5 in skeletal muscle remains unclear, and some functions have been indirectly proposed to occur in muscle by data extrapolation from other tissues. Thus, TGR5 can induce the deiodinase 2 expression, which would increase energy expenditure and oxygen consumption, based on studies previously described for brown adipose tissue, in a mechanism dependent on CREB activated by PKA [68]. In addition, it has been proposed that TGR5 expression can be upregulated in skeletal muscle under exercise and could favor myogenesis [69]. Accordingly to these antecedents, we have previously described that the TGR5 receptor increases its expression during skeletal muscle differentiation [47]. However, its participation in pathologies that affect skeletal muscle has been poorly studied. In this line of evidence, recent reports from our laboratory indicate that the absence of the TGR5 expression using a murine model of CCLD and knock-out (KO) for the TGR5 receptor prevents sarcopenia induced by CCLD. This antecedent suggests bile acids can mediate its effect on skeletal muscle through this receptor [47]. On the other hand, when incubating C₂C₁₂ myotubes with cholic acid (CA) and deoxycholic acid (DCA), a decrease in MHC protein levels was observed, together with an increase in MuRF-1 and atrogin-1 [47]. These alterations are molecular markers of a sarcopenic-like phenotype in vitro. In addition, when mitochondrial parameters were analyzed, a decreased mitochondrial mass, increased mitochondrial ROS levels, and reduced oxygen consumption rate was observed (Antioxidants in press). Therefore, bile acid treatment causes mitochondrial dysfunction in myotubes and isolated skeletal muscle fibers. However, the mechanisms that may be causing the abnormal functioning of the mitochondria have not been described in this model. Thus, we evaluated the effect of CA and DCA on processes that could regulate mitochondrial biomass, specifically mitogenesis and autophagy.

10.2 Methods

10.2.1 Cell Culture

C₂C₁₂ myoblasts were obtained from the American Type Culture Collection (ATCC). The media used for the expansion and maintenance of myoblasts and their differentiation into myotubes were Dulbecco’s Modified Eagle’s Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL of Penicillin, 100 µg/mL of Streptomycin, and 0.25 µg/mL of Amphotericin B (Gibco), supplemented with fetal bovine serum (FBS) (Hyclone) and horse serum (HS) (Thermo Fisher Scientific, Waltham, MA, USA) respectively. Cultures of C₂C₁₂ myotubes generated from differentiated myoblasts were used for all experiments [70]. Myoblasts were expanded and maintained by seeding them in
culture dishes in DMEM medium supplemented with 10% FBS. For experiments, 30,000 cells/cm² were seeded in DMEM-10% FBS. The next day, these cells were differentiated by washing the plate twice with PBS and DMEM. Finally, the culture was incubated with DMEM supplemented with 2% HS to differentiate, which was changed every 24 h until day 5 of differentiation, when cells were treated. Cell cultures for expansion and differentiation were grown at 37 °C, with 5% CO₂.

10.2.2 Treatments of C₂C₁₂ Myotubes

Myotubes from 5 days of differentiation were washed with 1 mL of DMEM and maintained in a 2% HS-DMEM medium. Then, cholic acid (CA, 300 μM) (Sigma-Aldrich, St Louis, MO, USA) or deoxycholic acid (DCA, 120 μM) (Sigma-Aldrich, St Louis, MO, USA) was added for 8, 24, 48, or 72 h (h). For autophagy assay, a pre-treatment with chloroquine (CQ, 50 μM) (Sigma-Aldrich, St Louis, MO, USA) was added 30 min (min) before the treatment with bile acids to visualize changes in LC3II levels at the 8 h. For the other treatment (48 and 72 h) in autophagy assay, CQ was added 8 h before finishing the treatment. The cultures designated as the control group (Vehicle) were incubated with 2% HS-DMEM and CQ [71]. Once the incubations were finished, the plate was washed twice with 1 ml of cold PBS and frozen at −20 °C until protein extraction was carried out.

10.2.3 Protein Extraction

At the end of experiments, C₂C₁₂ cells were incubated with RIPA buffer (50 mM Tris–HCl pH 7.5; 150 mM NaCl; 1 mM Na₂EDTA; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; phosphatase inhibitors: 1 mM orthovanadate; 1 mM NaF; 30 mM sodium pyrophosphate sodium; protease inhibitors: 1 mM PMSF and 1 mM cocktail of protease inhibitors) under orbital shaking for 15 min. Subsequently, the cell lysate was removed, recovered, and centrifuged at 2200 × g for 10 min at 4 °C. Finally, the supernatant was recovered, and the pellet corresponding to cell debris was discarded. Protein samples were stored at −20 °C for total protein measurement and subsequent use in Western blot assays. The total protein concentration in the extracts was quantified using the Micro BCA™ Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA).

10.2.4 Western Blot

30 μg of proteins were used to detect TFAM, LC3II, and LC3I. Protein samples were mixed with loading buffer (60 mM Tris–HCl pH 6.8; 25% glycerol; 2% SDS; 0.7 M β-mercaptoethanol; 0.1% blue strip bromophenol) in a 1:5 ratio of the volume to be loaded. Electrophoresis was then carried out under denaturant conditions in 10% acrylamide: bisacrylamide (29:1) gels (SDS-PAGE) to determine TFAM with running buffer (25 mM Tris–HCl pH 8.3; 192 mM Glycine; 0.1% w/v SDS) at 100 V and room temperature. To determine LC3II and LC3I, electrophoresis was performed on a 15% gel at 80 V and 4 °C. Subsequently, the proteins were transferred to a PDVF membrane Immobilon-P (ThermoFisher Scientific, Waltham, MA, USA), which was previously activated with methanol for 5 min. The transfer sandwich was assembled with the membrane and the gel, and they were placed in a chamber with transfer buffer (25 mM Tris–HCl pH 8.3; 192 mM Glycine; 20% v/v Methanol) and allowed to transfer at 350 mA for 90 min at room temperature, except for LC3 which was performed at 4 °C. After transference, the membrane was again immersed in methanol for 5 min and allowed to dry at room temperature. Next, the membrane was blocked with a solution of TBS (150 mM NaCl; 50 mM Tris–HCl pH 7.5) − 0.1% Tween 20–5% BSA for 1 h. In the end, the blocking solution was discarded to incubate the membranes with the primary antibodies in the blocking solution at 4 °C overnight. The following day, the membrane was washed three times for 5 min with TBS-0.1% Tween 20. The secondary
α-Rabbit antibody (Thermo Fisher Scientific, Waltham, MA, USA) was added and diluted in a TBS-0.1% Tween 20–5% BSA for 1 h at room temperature. Then, the membranes were washed three times with TBS-0.1% Tween 20, followed by three washes with PBS. Finally, the primary/secondary antibody complex’s binding to the target proteins was revealed by chemiluminescence (Thermo Fischer Scientific, Walthman MA, USA) and detected through an image documentation system FOTO/Analyst Luminary/FX Workstation (Fotodyne Inc., Hartland, WI, USA). The image quantification was carried out using the Image J program (NIH, Bethesda, MD, USA), and the protein levels, in the case of TFAM, were normalized to the levels of β-actin, used as load control. In the case of LC3II, the LC3I levels were used as standardization since the analysis performed was the LC3II/LC3I ratio. To calculate the autophagic flux, the following calculation was used [(LC3II + CQ) – (LC3II-CQ)].

10.2.5 Plasmids

A luciferase plasmid reporter ligated to a section of the PGC-1α gene promoter extracted from an Escherichia coli DH5α strain was used. PGC-1 alpha promoter 2 kb luciferase (pPGC-1α-Luc) was a gift from Bruce Spiegelman (Addgene plasmid # 8887; http://n2t.net/addgene:8887; RRID: Addgene_8887) [39]. Finally, together with pPGC-1α-Luc, a plasmid containing the Renilla luciferase gene (pRL-SV40) was co-transfected, which was used to normalize the luciferase activity. pRL-SV40 was a gift from Ron Prywes (Addgene plasmid # 27163; http://n2t.net/addgene:27163; RRID:Addgene_27163) [39].

10.2.6 Amplification and Transfection of Plasmids

The bacteria containing the reporter plasmid pPGC-1α-Luc were grown overnight (O.N.) in Terrific Broth liquid medium (Thermofisher Scientific, Waltham, MA, USA), supplemented with 100 µg/mL of Ampicillin as an antibiotic for selection. The following day, a protocol of DNA plasmid isolation and purification Maxi-Prep was performed using the E.Z.N.A® Plasmid DNA Maxi Kit (Omega Bio Tek, Norcross, GA, USA). Once the plasmid was purified, 0.5 µg/mL of it was digested with 0.25 U/µL of the enzyme BamHI (Thermofisher Scientific, Waltham, MA, USA) O.N. at 37 °C. An agarose gel was prepared by 0.7% m/v in Tris–acetate EDTA (TAE) buffer, and 5 µL of plasmid, mixed with loading buffer in a 5:1 ratio, was loaded and electrophoresed at 70 V for 1 h.

C2C12 cells were co-transfected with 1 µg of pPGC-1α-Luc and 0.02 µg of pRL-SV40 using 1 µl of LipofectAMINE 2000 (Thermofisher Scientific, Waltham, MA, USA) in Opti-MEM I. After 6 h, FBS was added to the medium, and the cells were cultured for 12 h. Further, the cells were differentiated for 4 days, and myotubes were incubated with DCA (120 µM) and CA (300 µM) for 24 h.

10.2.7 Luciferase Activity

After incubation with bile acids, the luciferase activity was determined using the reagents and the protocol of the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA). The DualGLO reagents and the GLOMAX™ 20/20 Luminometer (Promega, Madison, WI, USA) were used to detect luciferase activity. Each activity value Luciferase was normalized by its respective Renilla Luciferase activity value.

10.2.8 Statistical Analysis

The statistical analysis of the data was performed with Prism 9.0 analysis software (GraphPad Software, San Diego, CA, USA). The non-parametric test, “The Mann–Whitney U test,” was used for all the two groups’ studies. Differences were considered significant when the p-value was < 0.05.
10.3 Results

10.3.1 Cholic and Deoxycholic Acids Impair Autophagy in C₂C₁₂ Myotubes

Autophagy is a process that can be altered under sarcopenic conditions. For this reason, we evaluated autophagy parameters such as the LC3II/LC3I ratio through Western blot analysis in a model of sarcopenic-like phenotype in vitro using C₂C₁₂ myotubes. In this autophagy assay, chloroquine (CQ), a molecule that prevents autophagosome fusion with the lysosome [106], was used to determine the increase in the levels of LC3II. As shown in Fig. 10.1a, 16 KDa and 14 kDa bands are associated with LC3I and LC3II, respectively. The graph of Fig. 10.1b shows that the incubation of CA with CQ does not change the ratio of LC3II/LC3I at 8 h compared to the vehicle condition with CQ. However, cells treated for 48 h with CA plus CQ increased the LC3II/LC3I ratio by 1.34-fold (Fig. 10.1c, d) and by 1.28-fold at 72 h (Fig. 10.1d, f) compared to the CQ-treated vehicle. Therefore, treatment with 300 µM CA in the presence of CQ can increase the LC3II/LC3I ratio in C₂C₁₂ myotubes. In the case of the myotubes incubated with DCA, there is no increase in LC3II/LC3I at 8 h of treatment in the presence of CQ (Fig. 10.2a, b). In contrast, incubation for 48 h incremented 2.13-fold in the LC3II/LC3I ratio compared to the vehicle with CQ (Fig. 10.2c, d). At 72 h, there were no changes in the LC3II/LC3I levels compared to the same experimental groups (Fig. 10.2e, f). Therefore, the treatment with 120 µM of DCA increases LC3II/LC3I exclusively at 48 h.

From the results obtained, it was possible to determine that the treatment with CA decreases the autophagic flow. However, treatment with DCA induces a biphasic response with a transient increase at 48 h and further diminution at 72 h.

10.3.2 Cholic and Deoxycholic Acids Decrease PGC-1α Transcriptional Activity Without Altering TFAM Protein Levels in C₂C₁₂ Myotubes

PGC-1α is a master gene to induce mitogenesis, and an increment in its transcriptional activity is associated with increased mitochondrial biogenesis. Therefore, we used the reporter plasmid PGC-1α-Luc to determine whether bile acids affect the transcriptional activity of the PGC-1α promoter [83]. First, myoblasts were transfected with PGC-1α-Luc and incubated with horse serum, a stimulus that induces PGC-1α expression, showing a 120% increase in luciferase activity compared to the control (Fig. S1). Therefore, the transfected plasmid in the myoblasts can respond to a stimulus that induces the expression of PGC-1α.

C₂C₁₂ myotubes previously transfected with the plasmid PGC-1α-Luc were treated with CA and DCA for 24 h. CA and DCA (Fig. 10.4a, b,
respectively) caused a 50% decrease in the luciferase activity compared to the vehicle. Therefore, treatment with both bile acids decreases the transcriptional activity of the PGC-1α promoter.

Another protein involved in mitogenesis downstream of PGC-1α is TFAM. Thus, TFAM protein levels were analyzed by Western blot. Incubation with CA did not change TFAM levels at 8 h (Fig. 10.5a, b). However, CA increased 2.9-fold at 48 h after incubation (Fig. 10.5d, e). The same Figure shows that at 72 h, there were no changes under CA incubation (Fig. 10.5a, c). DCA incubation produced a transient upregulation of 1.67- and 1.5-fold in TFAM levels at 48 and 72 h, respectively (Fig. 10.5d, f), while no changes were observed at 8 h (Fig. 10.5a, c).

Together, these results indicate that both CA and DCA increase TFAM levels, which does not correlate with the decrease in PGC-1α promoter activity (Fig. S1).

10.4 Discussion

Autophagy is a process that eliminates cellular components (including organelles) that could be damaged and recycled [14]. The induction of autophagy pathways has been linked to sarcopenia [13]. The results in the present manuscript show that treatment with CA or DCA increases LC3II/LC3I ratio. This result indicates that these bile acids induce the conversion of
LC3I to LC3II, thereby increasing the autophagosome. However, when autophagic flux was analyzed, bile acids decreased this process. Thus, the increase in the LC3II/LC3I ratio obtained when treating myotubes with CA and DCA may be due to an accumulation of autophagosomes that are not degraded via the lysosome. Therefore, treatment with bile acids inhibits autophagy.

The published studies of bile acids and their effect on autophagy are limited to events in the hepatic tissue [72–74]. Under physiological conditions, after food intake, bile acids can act as nutrient sensors in the liver, promoting anabolic processes and inhibiting catabolic processes, including autophagy [75, 76]. Interestingly, bile acids-dependent inhibition of autophagy in the liver depends on the FXR receptor. This receptor is not expressed in skeletal muscle, discarding its participation in the inhibition of autophagy mediated by CA and DCA in myotubes observed in the present study [72]. Similar effects have been published using chenodeoxycholic acid (CDCA) in a human liver cell line, causing a decrease in autophagy [73]. Contrary to these antecedents, in a liver disease model caused by α1-antitrypsin protein deficiency, treatment with ursodeoxycholic acid (UDCA) induces autophagy through an unknown mechanism [74]. Therefore, our results about the autophagy induced by CA and DCA are essential as evidence of this process in a non-hepatic cell type.
Fig. 10.3 Cholic acid declines, and deoxycholic has a biphasic response, the autophagic flux in C<sub>2</sub>C<sub>12</sub> myotubes. C<sub>2</sub>C<sub>12</sub> myotubes were incubated with 300 µM of cholic acid (CA) (a–c) or 120 µM of deoxycholic acid (DCA) (d–f) for 8 (a–d), 48 (b–e), and 72 h (c–f) in absence or presence of chloroquine (CQ). At the end of the experiments, a Western blot of LC3I (16 KDa) and LC3II (14 KDa) was performed using β-actin (42 KDa) as the loading control. Images from Figs. 10.1 and 10.2 were used to calculate autophagic flux using \([\text{LC3II + CQ} - \text{LC3II-CQ}]\). The control condition (Vehicle) corresponds to myotubes without CA or DCA. Value of the graphs corresponds to the mean ± SEM of the groups normalized to vehicle and expressed as a fold of change. (n = 3, *p < 0.05, Mann–Whitney U test)

Fig. 10.4 Cholic and deoxycholic acids decreased PGC-1α transcriptional activity in C<sub>2</sub>C<sub>12</sub> myotubes. Undifferentiated C<sub>2</sub>C<sub>12</sub> cells were co-transfected with a plasmid reporter containing a section of PGC-1α promoter coupled to the luciferase gene and a pRL-SV40 plasmid. Cells were differentiated for 5 days and then incubated with 300 µM of cholic acid (CA) (a) or 120 µM of deoxycholic acid (DCA) (b) for 48 h. When the treatment was finished, dual luciferase activities were measured and expressed as a percentage of change. The control condition (Vehicle) corresponds to myotubes without CA or DCA. The graphs (a and b) value corresponds to the mean ± SEM of the groups normalized to vehicle. (n = 3, *p < 0.05, Mann–Whitney U test)
The absence of the FXR receptor in skeletal muscle, through which bile acids generate their anti-autophagic effect, and the presence of the TGR5 receptor suggest that it could participate in the autophagy inhibition mechanism. However, it must be further evaluated.

The life cycle of mitochondria allows the maintenance of a pool of healthy mitochondria in the cell. Among the processes involved in the life cycle is mitogenesis, which generates an increase in the number of new mitochondria and an increase in mitochondrial size and mass [36]. Mitochondrial biogenesis is repressed in sarcopenia, mainly associated with a decrease in the master regulator PGC-1α, which reduces the levels of all genes downstream [42, 45, 46, 77]. In the present study, using a transfection strategy of a plasmid reporter for the PGC-1α promoter

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**Fig. 10.5 Cholic and deoxycholic acids increase TFAM protein levels in C2C12 myotubes.** Differentiated C2C12 cells forming myotubes were incubated with 300 µM of cholic acid (CA) (a and b, d and e, g and h) or 120 µM of deoxycholic acid (DCA) (a, c, d, f, g, i) for 24 (a–c), 48 (d–f), and 72 h (g and i). When the treatment was finished, a Western blot of TFAM (24 KDa) was performed using β-actin (42 KDa) as the loading control. The control condition (Vehicle) corresponds to myotubes without CA or DCA. The value of the graphs (B–C, E–F, H–I) corresponds to the mean ± SEM of the groups normalized to vehicle and expressed as fold of change. (n = 3, *p < 0.05, Mann–Whitney U test)
coupled with the luciferase gene allowed us to analyze the behavior of its transcriptional activity in the presence of CA and DCA. Our results enable us to speculate that the expression of PGC-1α is decreased when myotubes are treated with these bile acids. Further experiments should be performed to evaluate the effect of CA and DCA on protein levels of PGC-1α, its intracellular location, and possible post-translational modifications that modulate its activity as a transcriptional coactivator and regulator of target genes that affect mitogenesis.

In addition, the effect of CA and DCA on TFAM protein, a target gene of PGC1-α, was evaluated [40]. Unexpectedly, an increase in TFAM protein levels by CA and DCA was observed. Thus, it is probable that a PGC1-α-independent mechanism can explain the increment in TFAM levels. A possible explanation for this phenomenon is the existence of an alternative pathway for the induction of TFAM levels. Studies in human aortic endothelial cells support this assertion, showing that HIF2α and c-Myc could regulate TFAM expression through a mechanism independent of PGC-1α and PGC-1β [78]. This possibility is reinforced by studies in cardiac myocytes that demonstrated mitochondrial biogenesis and markers such as TFAM were regulated in c-Myc-dependent and PGC-1α-independent ways [79]. Thus, a mechanism that modulates PGC-1α-independent mitogenesis involves the c-Myc protein. Subsequent experiments must be conducted to establish whether this mechanism could participate in regulating mitogenesis by CA and DCA. In this context, there is evidence that DCA and taurodeoxycholic acid (TDCA) can increase c-Myc mRNA levels [80–82]. Therefore, bile acids could induce c-Myc expression in C2C12 myotubes, increasing TFAM levels independent of PGC-1α. A recent publication from our group indicates that treatments with CA and DCA cause a decrease in mitochondrial biomass. This asseveration suggests that although TFAM levels increased with CA and DCA, more deep studies must be performed to evaluate the balance between mitogenesis and mitophagy to produce a decrease in mitochondrial mass.

TGR5 is the only high-affinity receptor for bile acids expressed by skeletal muscle cells. TGR5 is a G protein-coupled membrane receptor that, when activated by bile acids, increases cyclic AMP (cAMP) levels [64]. When TGR5 binds to its ligands and increases cAMP levels, promoting the activation of the "cAMP response element-binding" (CREB) transcription factor [83]. Interestingly, the mitogenesis master regulator gene, PGC-1α, has CREB binding sites in its promoter. Therefore, CREB activation should induce PGC-1α expression [84]. It has been reported in an in vitro model of endothelial cells from the aorta and human podocytes that the activation of TGR5 by taurolithocholic acid (TLCA) and the specific activator INT-777 induces an increase in PGC-1α levels together with markers of mitochondrial biogenesis, dependent on CREB activation [85, 86]. If the effects of CA and DCA observed in our work are dependent on TGR5, the question that arises is how the activation of this receptor by CA and DCA generates an opposite effect (decrease in biomass, mitochondrial dysfunction) to that described in other cell types [85, 86]. Further studies must be performed to elucidate this question.

10.5 Conclusions

Cholic and deoxycholic acids increased LC3II/LC3I ratio by inhibiting the autophagic flux in C2C12 myotubes. Cholic and deoxycholic acids decreased PGC-1α promoter activity in C2C12 myotubes with increased TFAM protein levels.

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Upregulation of CCL5/RANTES Gene Expression in the Diaphragm of Mice with Cholestatic Liver Disease

Vania Morales, Andrea González, and Claudio Cabello-Verrugio

Abstract

Chronic liver diseases are a group of pathologies affecting the liver with high prevalence worldwide. Among them, cholestatic chronic liver diseases (CCLD) are characterized by alterations in liver function and increased plasma bile acids. Secondary to liver disease, under cholestasis, is developed sarcopenia, a skeletal muscle dysfunction with decreased muscle mass, strength, and physical function. CCL5/RANTES is a chemokine involved in the immune and inflammatory response. Indeed, CCL5 is a myokine because it is produced by skeletal muscle. Several studies show that bile acids induce CCL5/RANTES expression in liver cells. However, it is unknown if the expression of CCL5/RANTES is changed in the skeletal muscle of mice with cholestatic liver disease.

We used a murine model of cholestasis-induced sarcopenia by intake of hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC diet), in which we detected the mRNA levels for ccl5. We determined that mice fed the DDC diet presented high levels of serum bile acids and developed typical features of sarcopenia. Under these conditions, we detected the ccl5 gene expression in diaphragm muscle showing elevated mRNA levels compared to mice fed with a standard diet (chow diet). Our results collectively suggest an increased ccl5 gene expression in the diaphragm muscle concomitantly with elevated serum bile acids and the development of sarcopenia.

Keywords

Sarcopenia · Cholestasis · Myokine · CCL5/RANTES · Bile acids

Abbreviations

ALT Alanine aminotransferase
ALP Alkaline phosphatase
BA Bile acids
BIA Bioelectrical impedance analysis
CCLD Chronic cholestatic liver diseases
CLD Chronic liver diseases
CSA Cross-section area
CST 30-second (30-s) chair stand test
DDC Hepatotoxin
3,5-diethoxycarbonyl-1,4-dihydrocollidine

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11.1 Introduction

The skeletal muscle represents between 40 and 50% of the body mass and is an excellent reservoir of proteins since approximately 80% of the muscle fibers are composed of them [1]. Skeletal muscle participates in several mechanical functions, such as the generation of muscle strength and mobility. Skeletal muscle is composed of muscle fibers which are long and multinucleated cells. The contractile function is carried out by the presence of a sarcomere in the cytoplasm [1].

The sarcomere is the basic contractile unit of muscle fiber. Each sarcomere is composed of, at least, two central protein filaments: actin and myosin. These proteins form two types of myofilaments, thick and thin. Thick filaments are composed of myosin heavy chain (MHC) protein, whereas thin filaments are constituted by α-actin. Thin filaments also contain regulatory proteins, troponin, and tropomyosin [2]. The myofilaments are the active structures responsible for muscular contraction, which is described accordingly to the most popular model called the sliding filament theory. In this theory, active force is generated as actin filaments slide on the myosin filaments, resulting in the contraction of an individual sarcomere. In brief, muscular contraction is produced when actin-myosin cross-bridges are formed. This interaction occurs when a thick myosin filament containing numerous heads is attached to the thinner actin filaments. Basically, a myosin head is like a cocked spring, which flexes and produces a power stroke on binding with an actin filament. The power stroke slides the actin filament on the myosin, resulting in force generation and shortening of an individual sarcomere. Because sarcomeres are structures arranged in series by joining end to end throughout an entire muscle fiber, their simultaneous contraction shortens the muscle as a whole [3–5].

When myofilaments are viewed under an electron microscope, their arrangement gives the appearance of alternating bands of light and dark striations. A Z-band borders it on each end with adjacent I-bands, and there is a central M-line with adjacent H-bands and partially overlapping A-bands. The Z-band (or Z-disk) is a dense fibrous structure made of actin, α-actinin, and other proteins. Thin filaments (or actin filaments) are anchored at one end at the Z-band. Titin is anchored to both the Z-band and the M-line. Thick filaments are anchored in the middle of the sarcomere at the M-line. The I-band is the region on either side of a Z-disc that contains only thin filaments and titin. This partial overlap in filaments makes the A-band darker at its ends, leaving a light area in the middle (H-band) where there is no overlap with the light bands. A key clue to the mechanism of contraction was the finding that during contraction, the H and I bands shorten, while the A bands do not [2–4].

Skeletal muscle fibers are broadly classified as “slow-twitch” (type 1) and “fast-twitch” (type 2). Based on differential myosin heavy chain (MYH) gene expression, fast-twitch fibers are further classified into three major subtypes (types 2A, 2X, and 2B, although humans do not appear to have MYH4-expressing type 2B fibers).
Hybrid MYH expression in different fibers of a muscle group can allow for even more subtypes (1/2A, 2A/2X, 2X/2B), resulting in an almost continuous range of ATP usage and muscle contraction speeds, from the fastest (type 2B) to the slowest (type 1). Skeletal muscle fibers also vary in energy production. Type 1 and 2A fibers primarily use oxidative metabolism, and type 2X and 2B fibers rely on glycolytic metabolism. However, even here, there is variation, and energy usage is not a strict predictor of fiber type [6, 7].

The skeletal muscle plays an essential role in many activities, such as maintaining posture, speaking, breathing, generating muscle power, and moving the body. It has high plasticity and an endocrine function since it can produce and secrete molecules called myokines into circulation. Myokines are cytokines or peptides synthesized and released by fibers in muscle tissue in response to muscular contractions [8]. The term “myokine” was introduced by a Swedish scientist, Bengt Saltin, in 2003 [9]. Myokines are implicated in the autocrine regulation of metabolism in muscles and the para/endocrine regulation of other tissues and organs, including the adipose tissue, liver, bone, and brain, through their receptors [10, 11]. Some of the myokines reported in the literature are Irisin, interleukin 6 (IL-6), myostatin, and Insulin-like growth factor-1 (IGF-1) [12–15]. Irisin is a novel exercise-triggered myokine [16]. It is a peptide of 12 kDa mainly synthesized and secreted by skeletal muscle and a small amount by the liver and adipose tissue [17, 18]. To date, the irisin receptor is unknown. However, it is described that the target cells for irisin are white adipocytes, myocytes, and hepatocytes [19]. Thus, irisin communicates the skeletal muscle with adipose tissue [16, 20, 21]. In skeletal muscle, irisin induces myogenesis and mitochondrial biogenesis and protects against muscle atrophy [22, 23]. Myostatin, also known as growth differentiation factor 8 (GDF8), is a myokine belonging to the transforming growth factor-beta (TGF-b) superfamily, present in cardiac muscle and adipose tissue but is most abundant in skeletal muscle [24]. Myostatin is a negative regulator of muscle mass. In this line, the absence of the myostatin gene produces an increase in muscle mass, contributing to hypertrophy [25–27]. Interleukin-6 (IL-6) is a pro-inflammatory cytokine synthesized by several organs and is responsible for the acute phase reaction in the inflammatory process in injury [28, 29]. Under normal conditions, IL-6 levels in circulation are low, but in inflammatory states, these levels can rise above 1000-fold [30]. Furthermore, IL-6 has a critical metabolic role in controlling body weight, liver physiology, and bone metabolism [30]. IL-6 can have two opposite functions in skeletal muscle: pro-inflammatory or anti-inflammatory activities. IGF-1 is a myokine with a single-chain polypeptide of 70 amino acid residues cross-linked by three disulfide bridges and a molecular weight of 7 KDa [31]. IGF-1 is synthesized and secreted mainly in the liver and skeletal muscle, acting on multiple target tissues in an endocrine/paracrine and autocrine manner. In skeletal muscle, increased IGF-1 levels after endurance and resistance training favors cell growth and differentiation by stimulating anabolic pathways activity and decreasing the catabolic pathway activity [28, 32–34].

There are other myokines less investigated, such as CCL5/RANTES. This myokine, also called chemokine ligand 5 (Regulated upon Activation, Normal T cell Expressed and Secreted), belongs to the CC subfamily of chemokines since it has a conserved region of 4 cysteine amino acids in its structure, and it has a size of 7.4 kDa [12–15]. Most inflammatory cells can express CCL5/RANTES. T cells and monocytes are the most common types of CCL5-expressing cells. While CCL5/RANTES can bind to CCR1, CCR3, CCR4 and CCR5, it has the highest affinity to CCR5 [35–37]. CCR5 (also known as CD195) is a G-protein-coupled receptor (GPCR) whose transcription is regulated by CREB-1. CCR5 contains 352 amino acids with a calculated molecular mass of 40.6 kDa and shares 71% sequence identity with CCR2, with most of the differences being located in the extracellular and cytoplasmic domains [38–41]. Both receptors are in proximity on chromosome 3p21 [39, 40].
addition to T cells, CCR5 expresses in smooth muscle endothelial cells, epithelial cells, and parenchymal cells [36, 42]. Besides CCL5/RANTES, CCR5 also binds to CCL3 (MIP-1α) and CCL4 (MIP-1β) with an N-terminal extracellular tail [43]. CCR5 is the most important receptor for HIV-1 infection with a gp-120 combination. Thus, it has been considered a promising target for anti-HIV therapies [44]. Regarding HIV, the predominant role of CCR5 for viral entry and replication is depicted by the resistance to HIV-1 infection of individuals who lack CCR5 due to a 32-bp deletion in the CCR5 gene [45, 46]. Many (nonfunctional) CCR5 variants were identified in various human populations in addition to the deletion of 32 bp, suggesting that this receptor has been subject to unknown selective forces [47, 48].

CCL5/RANTES participates in the inflammatory process and is expressed mainly by platelets, macrophages, endothelial cells, and the skeletal muscle [37, 49, 50]. In hepatic fibrosis, it has been observed in a murine model that there is an increase in the intrahepatic expression of CCL5/RANTES and that by eliminating the gene (ccl5−/−), fibrosis decreases. This effect was also observed when an antagonist was administered for CCL5/RANTES (Met-CCL5) [51]. In skeletal muscle, antecedents in a murine model with free access to a running wheel showed a reduction in the protein levels of CCL5/RANTES and the ccl5 gene expression in muscles compared to muscles from sedentary mice [52]. Another study using an “in vitro contractile system” model in C2C12 myotubes applying electrical pulses to stimulate muscle contraction showed a decrease in the expression of CCL5/RANTES after electrical stimulation [53].

The skeletal muscle can be affected by several chronic non-transmissible diseases, such as chronic liver diseases (CLD). These pathologies are a group of disorders characterized by decreased liver function resulting from chronic inflammation or damage to liver tissue [54, 55]. CLD are highly prevalent pathologies, with 5.5 million people suffering from them worldwide [56]. In Chile, according to data from the Department of Statistics and Health Information (DEIS) of the Ministry of Health, in 2011, a total of 94,985 deaths, 5.2%, corresponding to liver pathologies, ranking after ischemic pathologies heart and cerebrovascular. In CLD, the liver progressively deteriorates, beginning with an inflammatory process that becomes chronic and develops early fibrosis due to an increase in the extracellular matrix components. Subsequently, and due to this chronic inflammation and fibrosis, the liver is permanently damaged, losing most of its functions irreversibly, a condition known as liver cirrhosis, which corresponds to the final stage of the pathology and in which patients may need a liver transplant [57, 58]. Liver cirrhosis has one of the highest mortality rates in Latin America, with 24.2% hospitalized in the general ward, which increases to 86% in intensive care unit patients [59–61]. The most common causes of CLD are chronic viral hepatitis, alcohol abuse, biliary cirrhosis, and non-alcoholic fatty liver disease (NAFLD) [62]. NAFLD includes a variety of liver disorders, such as non-alcoholic fatty liver, non-alcoholic steatohepatitis (NASH), cirrhosis, and NASH associated with hepatocellular carcinoma [63]. Another type of CLD that is highly prevalent is that of cholestatic origin, called chronic cholestatic liver diseases (CCLD). Cholestasis is an impairment in bile formation and/or bile flow, which might present clinically with fatigue, pruritus, and jaundice. Cholestasis can be classified as intrahepatic or extrahepatic. Intrahepatic cholestasis can occur due to a functional hepatocellular defect or obstructive lesions of the intrahepatic biliary tracts distal to or from bile canaliculi (intrahepatic biliary tract distal from bile canaliculi). Extrahepatic biliary obstruction can be caused by stones, tumors, cysts, or strictures [64].

Among the complications that CCLD entails, we find dyslipidemia, portal hypertension, and mainly the loss of muscle mass and strength and physical function associated with mobility, a condition known as sarcopenia [56, 65, 66]. Sarcopenia is defined by typical features such as decreased skeletal muscle strength and mass and low physical performance [67]. Sarcopenia is considered secondary when related to nutritional alterations, immobilization, and chronic non-
transmissible diseases [68, 69]. Sarcopenic skeletal muscle shows a decrease in cross-section area (CSA), muscle fiber type switching, and decreased levels of sarcomeric proteins, such as tropomyosin, myosin heavy chain (MHC), and troponin I [70]. Mechanistically, alterations in the ubiquitin–proteasome system, such as increased expression of E3 ubiquitin ligases atrogin-1 and MuRF-1, oxidative stress, typically denoted by increased production of reactive oxygen species (ROS) and redox-dependent protein modifications, mitochondrial dysfunction, autophagy, and myonuclear apoptosis [55, 70].

Sarcopenia is one of the most critical and prevalent comorbidities related to the development and progression of CLD, including CCLD [71–74]. Muscle dysfunction is associated with increased morbidity and mortality in patients with CLD, alterations in typical daily activities, and frailty, disability, and hospitalization [73, 75–77]. Interestingly, sarcopenia has been reported as an independent predictor of pre- and post-liver transplantation mortality [78]. The prevalence of sarcopenia in CLD is high, and it has been shown that there is a loss of muscle mass in the early and late stages of the pathology, a condition that worsens proportional to its severity [72, 74]. In liver cirrhosis, the prevalence is 48.1%, and in the final stages of CLD, about 60% of patients present it, which is associated with decompensation and higher morbidity and mortality [72, 75, 76]. Furthermore, people with sarcopenia, after liver transplantation, have a higher risk of infection, a more extended stay in Intensive Care Units (ICU) and the need for connection to mechanical ventilation, and a higher rate of post-surgery complications (respiratory, kidney, heart, and graft failure) than those without sarcopenia [79–82]. It is also associated with an increased risk of falls, fractures, lower health-related quality of life, acute decompensation of liver disease, acute on chronic liver failure, and increased risk of death in patients with cirrhosis [69, 83, 84]. Unfortunately, sarcopenia is not reversible after liver transplantation. In fact, it may increase due to the use of immunosuppressive drugs such as steroids and calcineurin inhibitors. This situation can favor the appearance of the multiple complications already mentioned, hence the relevance of being diagnosed and treated on time [85, 86].

The diagnosis of sarcopenia must consider the evaluation of the three parameters that compose it: muscle strength, muscle mass, and physical function associated with mobility. Muscular strength is defined as the force generated by muscular contraction against an external load [87]. Clinically, it can be evaluated by grip strength, chair stand test, 1 or 10 repetition maximum (1 RM or 10 RM). Muscle mass is the part of the total body mass composed of musculoskeletal tissue [88]. Muscle mass can be indirectly evaluated with imaging and clinical methods such as Dual-energy X-ray Absorptiometry (DXA), Computed tomography, Magnetic resonance imaging (MRI), Ultrasound (US), Bioelectrical impedance analysis (BIA), and Anthropometry and calf circumference. Physical function is defined as the function of the entire body related to mobility and locomotion, which allows the performance of functional physical activities in daily life independently [69, 89, 90]. Usually, it is evaluated with tests that involve displacement, such as Gait speed, Timed-Up and Go test (TUG), 6-min walk test, 400 m walk test, or with tests that require the lower extremities, such as Short Physical Performance Battery (SPPB) or 30-s (30-s) chair stand test (CST).

Our previous work on cholestatic CLD-associated sarcopenia using a murine model by intake of hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) presents the typical features of sarcopenia, such as decreased muscle strength and mass, and decline in muscle function. This model of sarcopenia is characterized by a fiber-type transition, a reduction in fiber diameter, and MHC levels. We also found oxidative stress evaluated by increased ROS levels and carbonylated- and 4HNE-modified proteins. In addition, changes in proteostasis were determined. Specifically, the high levels of MAFbx/atrogin-1 and MuRF-1/TRIM63, two E3 ubiquitin ligases associated with muscle wasting [55, 66]. Sarcopenia is a crucial determinant of
frailty that leads to loss of autonomy and functionality in activities of daily living and is an independent predictor of post-liver transplant mortality [91–93].

Among the contributors to sarcopenia in CLD are bile acids. In cholestatic EHC generated by occlusion of the bile ducts, there is an increase in plasma bile acids levels, affecting not only the liver but also other organs and tissues in the body, such as the gut, brown adipose tissue, macrophages, and also skeletal muscle, mainly due to their functions as extrahepatic signaling molecules [94–97]. This increase in bile acids in the plasma reaches the skeletal muscle and, through the TGR-5 receptor located on the plasma membrane of skeletal muscle cells, induces a condition of sarcopenia in a DDC murine model. In addition, bile acids, specifically deoxycholic and cholic acids, cause the loss of muscle mass in cell cultures of C2C12 myotubes and isolated muscle fibers, leading to a sarcopenic-like phenotype [94]. Interestingly, bile acids can increase the ccl5 mRNA levels in hepatic cells [98, 99]. However, the modulation of the ccl5 gene expression in skeletal muscle is unknown under cholestatic conditions characterized by high levels of plasma bile acids.

11.2 Methods

11.2.1 Animals

C57BL/6 J WT male mice (16 weeks old) were randomized and separated into experimental groups to perform three independent experiments. Mice were fed with a standard diet (chow) or a diet supplemented with 0.1% 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) hepatotoxin to induce CLD (Sigma-Aldrich, St Louis, MO, USA) for six weeks [55, 100]. Each experimental group contained five to nine mice. The animals (weighing 25–30 g) were kept in the mice housing of the Department of Biological Sciences at Universidad Andrés Bello, at a controlled temperature (22–24 °C), with food and water ad-libitum and with inverted 12-h light/dark cycles (Light: 8 PM to 8 AM). The animals were adapted for two weeks before the start of the experimental intervention to ground food and five days before the evaluation tests that require acclimatization, such as the treadmill. After treatment, the animals were anesthetized with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine and sacrificed through cervical dislocation. Subsequently, the gastrocnemius and tibialis anterior muscles were dissected, cleaned from residual non-muscular tissue, and removed from animals. The muscles used to obtain the total tissue homogenate used in RNA extraction were quickly frozen in liquid N2 and stored at −80 °C until processing. Muscles used for histological analysis were frozen promptly in isopentane previously cooled in liquid N2 and stored at −80 °C until processing.

Our experiments followed all international, national, and institutional suggestions and guidelines for animal care and use. Our studies and procedures with animals had the Animal Ethics Committee’s formal approval at the Universidad Andrés Bello (approval number 007/2016, March 2016).

11.2.2 Plasma Bile Acids Levels

Total plasma bile acids levels were using the colorimetric assay (Randox Laboratories Ltd., Kearneysville, WV, USA). Blood was drawn from the mouse’s tail (approximately 30 µL) and centrifuged at 1500 × g for 10 min at 4 °C. Subsequently, the plasma was separated for the assay [101].

To determine the concentration of total bile acids, a calibrator of known concentration was used, which was resuspended in physiological saline (0.9% NaCl). Using 750 mL of reagent 1 and 250 mL of reagent 2 provided by the kit were added, and subsequently, 10 µL of calibrator or mouse serum. The respective blanks were used, and the OD405nm was measured in plastic cuvettes. Thus, the absorption of the samples and the calibrator provided by the kit were measured at 405 nm at 60 and 120 s (A1 and A2, respectively), and the difference between
the two values was calculated ($\Delta A = A_2 - A_1$). The total bile acid concentration is calculated based on the following formula:

\[
(\Delta A_{\text{sample}}/\Delta A_{\text{gauge}}) \times [\text{gauge}] = [\text{sample}] \mu\text{mol/L}
\]

### 11.2.3 Parameters of Liver Injury

The colorimetric assay (Sigma-Aldrich, St Louis, MI, USA) was used to measure serum alanine aminotransferase (ALT). Serum alkaline phosphatase (ALP) activity and total bilirubin levels were determined by routine clinical chemistry testing, as previously described [102].

### 11.2.4 Hepatomegaly

Hepatomegaly is one of the data that allows for characterizing and confirming liver damage induced by DDC hepatotoxin, where we observed an increase in liver weight in animals with CLD [95].

### 11.2.5 Measurement of Muscle Mass

The muscle mass was measured as the lean mass of mice by EchoMRI resonator analysis (Echo Medical Systems, Houston, TX, USA) at six weeks of treatment.

### 11.2.6 Maximal Incremental Exercise Test

To assess physical performance, mice were subjected to a maximal incremental race test on a treadmill (Panlab LE8610MTS). Briefly, mice were on a treadmill starting at 12 cm/s for 2 min. Then, velocity was increased up to 15 cm/s for 3 min: Further, an increment of 2 cm/s each 1 min until the fatigue of mice was determined by the lack of response to the motivational stimulus (air puff) for 10 consecutive seconds [103].

### 11.2.7 Grip Strength Test

To evaluate the muscle strength in the forelimb and hindlimb, we used a dynamometer for little animals (Ugo Basile, Grip Strength meter 47,200). The procedure was carried out according to previous reports until it reached 15 repeats in the hind and forelegs of animals, which allows for calculating the maximum strength of these. The final value was expressed as the maximum force of the hind and forelegs normalized by the length of the tibia of the animals [104, 105].

### 11.2.8 RT-qPCR

The diaphragm muscles were obtained after dissection to determine the expression of CCL5/RANTES in control and CLD animals. The samples were rapidly frozen and stored at $-80 \, ^\circ\text{C}$ until processing. For RNA extraction, a homogenate was obtained from 10 mg of tissue with 1 mL of Chomczynski-phenol reagent (Winkler, Chile) for 1 min with ultraturrax Tissue Tearor TM (Biospec Products Inc., Mexico) on ice. This homogenate was centrifuged at 16,000 g for 15 min at 4 $^\circ\text{C}$, and the resulting supernatant was mixed with chloroform (Merck, Germany) and again centrifuged at 16,000 g for 15 min at 4 $^\circ\text{C}$. The resulting aqueous phase was mixed with an equal volume of isopropanol (Merck, Germany) and allowed to precipitate overnight at $-20 \, ^\circ\text{C}$. At the end of the incubation, the sample was centrifuged at 16,000 g for 15 min at 4 $^\circ\text{C}$, the supernatant was removed, and the precipitate was washed with 75% ethanol in DEPC water (Winkler, Chile). Subsequently, it was centrifuged at 16,000 g for 15 min at 4 $^\circ\text{C}$. The supernatant was removed, and the precipitate was allowed to dry and resuspended in DEPC water. The supernatant with the mRNA was stored at $-80 \, ^\circ\text{C}$ until it was used for total RNA quantification [106].

Total RNA was quantified in the UV-mini-1240 spectrophotometer (Shimadzu, Japan) at 260 nm and 280 nm wavelength to verify RNA purity, using the 260/280 ratio, taking a value of
2.0 as a reference. Integrity was verified by electrophoresis in an agarose gel (1% agarose, 5% formaldehyde, MOPS buffer pH 7.2, in DEPC water), using MOPS in DEPC water as a running buffer [106].

Complementary DNA (cDNA) synthesis was performed from 1 μg of total RNA, which was incubated with DNase (2 U) in 1 × DNase buffer (Fermentas, Thermo Fisher Scientific, USA) at 37 °C for the digestion of possible genomic DNA traces. DNase was then inactivated by incubation in 50 mM EDTA solution for 10 min at 65 °C. Reverse transcription was performed with 25 mM random primers (Invitrogen, Thermo Fisher Scientific, USA), First Strand 1 × buffer (Invitrogen, Thermo Fisher Scientific, USA), 25 mM dNTP (Fermentas, Thermo Fisher Scientific, USA), Riboblock (10 U) (Fermentas, Thermo Fisher Scientific, USA), 0.1 M DTT (Invitrogen, Thermo Fisher Scientific, USA) and the enzyme MMLV (Moloney Murine Leukemia Virus Transcriptase Reverse) (100 U) (Invitrogen, Thermo Fisher Scientific, USA) in DEPC water. Reverse transcription was performed in the MultiGene Opti-Max thermocycler (Labnet International, USA). The cDNA obtained was stored at −20 °C until later use [106]. The cDNA from reverse transcription was analyzed to evaluate the ccl5 gene expression (Forward: ATCTCTGCAGCTGCCC TCAC; Reverse: CTTGGCGGTTCCTTCGAG TG) with SyBR Green using β-actin (Forward: GTGACGTTGACATCCGTAAAGA; Reverse: GCCGGACTCATCGTACTCC) as a housekeeping gene. PCR was performed in triplicate using an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). The mRNA expression was calculated using the comparative ΔΔ Ct method [107].

11.2.9 Statistical Analysis

The statistical analysis of the data was performed with Prism 9.0 analysis software (GraphPad Software, San Diego, CA, USA). The normality of the data was determined with the Shapiro–Wilk test. Normal data were analyzed with a t-test to compare the two groups. Differences were considered significant when the p-value was < 0.05.

11.3 Results

We corroborated the induction of cholestatic liver disease in mice that intake DDC hepatotoxin in the diet, measuring parameters of hepatic function as previously reported [55]. Figure 11.1a shows that mice fed a DDC diet for six weeks presented hepatic alterations evidenced by higher serum alanine aminotransferase (ALT) activity than mice fed with a chow diet. Figure 11.1b shows that mice from the DDC group have more elevated serum alkaline phosphatase (ALP) activity than the Chow group. In addition, we observed that animals fed with a DDC diet showed hepatomegaly (Fig. 11.2). These parameters indicate hepatic dysfunction. Then, we corroborated that mice fed with the DDC diet presented elevated plasma bile acid levels than mice fed with the chow diet (Fig. 11.3).

Together, these results demonstrated that mice fed a DDC diet developed a cholestatic liver disease with typical liver dysfunction and increased plasma bile acids levels.

Then, we evaluated the parameters of sarcopenia in mice fed with chow and DDC diets. First, we assessed the effect of the DDC diet on muscle strength through a grip strength test. Figure 11.4 shows that muscle strength is decreased in the forelimb (Fig. 11.4a) and hindlimb (Fig. 11.4b) in mice fed with the DDC diet compared to mice with the chow diet.

Another parameter key to describing sarcopenia is the muscle mass determined by nuclear magnetic resonance analysis. Figure 11.5 shows that the DDC group decreased muscle mass compared to the Chow group.

Further, we evaluated the physical function through the maximal incremental race test on a treadmill. Figure 11.6 shows that mice fed with the DDC diet presented an impaired physical function compared to mice fed with the chow diet. Thus, the DDC group had a decreased
maximum running distance (Fig. 11.6a), reduced maximum running speed (Fig. 11.6b), and decreased maximum race time (Fig. 11.6c) compared to the chow group.

Together, the results from muscle strength and mass and physical function confirm that mice fed the DDC diet developed sarcopenia.

Finally, we evaluated the ccl5 gene expression in diaphragm muscle from chow and DDC mice. Figure 11.7 shows that the mRNA levels for ccl5 increased in the diaphragm of the DDC group compared to the chow group. Thus, our results show that the diaphragm from cholestasis-induced sarcopenic mice presented elevated ccl5 gene expression, which correlated with plasma bile acids and liver dysfunction.

**Fig. 11.1** Mice with cholestatic liver disease presented alteration of hepatic parameters. C57BL/6 J male mice were fed a chow or a DDC-supplemented diet for 6 weeks. Blood was collected at the end of the experiment. a ALT and b ALP activities were measured. The values correspond to the mean ± SEM (n = 6–9). Statistical differences were examined by unpaired Student’s t-test and are indicated by p < 0.0001 with a 95% CI

**Fig. 11.2** Cholestatic liver disease produces hepatomegaly in mice. C57BL/6 J male mice were fed a chow or a DDC-supplemented diet for 6 weeks. After euthanasia, liver weight was obtained at the end of the experiments. The values correspond to the mean ± SEM (n = 6–9). Statistical differences were examined by unpaired Student’s t-test and are indicated by p < 0.0001 with a 95% CI

**Fig. 11.3** Mice affected with cholestatic liver disease have elevated plasma bile acid levels. C57BL/6 J male mice were fed a chow or a DDC-supplemented diet for 6 weeks. Blood was collected at the end of the experiment, and plasma bile acid levels were measured. The values were normalized to the chow diet and are expressed as the mean ± SEM (n = 6–9). Statistical differences were examined by unpaired Student’s t-test and are indicated by p < 0.0001 with a 95% CI
In the present study, we have observed that mice fed with DDC have elevated ccl5 gene expression in diaphragm muscles which correlated with high levels of bile acids, CLD, and developed muscle dysfunction.

An essential characteristic of the skeletal muscle is its high plasticity, allowing it to adapt to different stimuli, which can be positive (such as specific exercises or high-protein diets) or negative (such as the aging process, malnutrition, disuse, and chronic pathologies such as is cholestatic CLD).

In animals with CLD, we observed a decrease in muscle strength, muscle mass, and physical function compared to animals that received a standard diet (Chow) and evaluated by grip strength test (fore and hind limb strength), nuclear magnetic resonance (muscle mass) and maximum running capacity (physical performance). This sarcopenia is also observed in humans and is one of the main consequences of CLD, negatively affecting the physical function related to health and the performance in activities of daily living in patients who suffer from it. It is also an independent predictor of post-liver transplant mortality [91–93]. If we consider the scarcity of organs available for organ transplantation in Chile and the world, preventing sarcopenia or determining its presence and severity could help to choose those people who require the procedure and whose probability of success and survival is greater [78].

On the other hand, in cholestatic CLD, there are increased bile acids in the plasma, producing...
a deleterious effect on the liver, also affecting the skeletal muscle, decreasing its mass and strength, concomitantly with a decreased physical function, a condition referred to as sarcopenia.

Given the importance of the effect of bile acids on the skeletal muscle during EHC, in this research, we evaluated the concentration of bile acids in the serum of mice after finishing the treatment with the DDC diet in the sixth week of the experimental intervention. Our results confirm that the DDC animals presented higher plasma bile acid levels than mice fed a chow diet, as we have previously reported. The DDC hepatotoxin crystallizes in the bile ducts, producing their obstruction and generating an increase in bile acids in the plasma, which is consistent with the state and progression of the hepatic dysfunction in the mice. Additionally, bile acids have extrahepatic functions acting as signaling molecules and producing harmful effects in several tissues [94–97]. In skeletal muscle, bile acids can bind to the membrane TGR5 receptor to

Fig. 11.6 Cholestatic liver disease decreased the physical performance of mice. C57BL/6 J male mice were fed a chow or a DDC-supplemented diet for 6 weeks. At the end of the experiments, a maximal incremental run test was performed to measure a the decrease in the maximum running distance (m), b the decline in maximum running speed (cm/s), and c the reduction in maximum race time (s) in DDC group to compare chow group. The values correspond to the mean ± SEM (n = 6–9). Statistical differences were examined by unpaired Student’s t-test and are indicated by p < 0.0001 with a 95% CI.
generate atrophy and sarcopenia [94]. We have previously reported the mechanisms involved in the sarcopenia induced by the DDC diet, including activation of sarcopenic-specific E3 ligases MuRF-1 and atrogin-1, oxidative stress, unbalanced proteostasis of sarcomeric components, mitochondrial dysfunction, and myonuclear apoptosis. Interestingly, most of these features present in DDC-induced sarcopenia are reproducible by induction of sarcopenic-like phenotype mediated by bile acids in muscle fibers showing the critical role of bile acids in muscle dysfunction [94, 108].

Hepatomegaly also allows us to characterize and confirm liver damage induced by DDC hepatotoxin, and our results showed that the DDC mice had hepatomegaly. This increased liver weight could be due to chronic inflammation of the organ, dilatation of the bile ducts, protoporphyrin plugging, and a progressive change in liver tissue due to fibrosis and cirrhosis that can affect the size and weight of the organ [109].

Our results show that mice fed with a DDC diet presented increased ccl5 gene expression in the diaphragm muscle for the first time. This result is relevant because the first report correlates the ccl5 expression with a sarcopenic condition such as cholestatic liver disease.

CCL5/RANTES is a molecule that can be described as either a myokine or a cytokine. The latter has been described as having a role in regulating the immune system, where it has been observed in damaged liver tissue. A deficiency of CCL5/RANTES promotes better repair, helping a better inflammatory process and regeneration. Still, these effects have not been studied in their role as a myokine in muscle [110].

CCL5/RANTES is a new myokine with functions poorly described in skeletal muscle. The antecedents describe that ccl5 expression is modulated by contractile activity, decreasing these levels by contraction [53]. To date, the only precedent that relates an increase in the expression of ccl5 and the diaphragm muscle is in a model of endotoxemia and pseudomonas lung infection [111]. Interestingly our results were obtained in the diaphragm, a skeletal muscle with continuous and involuntary contractile activity, which could mask the more significant upregulation of ccl5 expression in other muscles with voluntary and non-continuous contractile activity.

Bile acids are excellent candidates for the soluble factor that could induce the upregulation of ccl5 gene expression. Some reports indicate that bile acids such as cholic, deoxycholic, and chenodeoxycholic acids can induce ccl5 expression [98]. In addition, some reports demonstrated that the increase of the ccl5 expression could be mediated by the activation of the NF-kB signaling pathway [99]. Interestingly, NF-kB is a signaling pathway involved in the induction of sarcopenia by several conditions [112–114]. In turn, it has been described that the NF-kB signaling pathway is activated in response to a variety of stimuli such as infections, exposure to cytokines, and growth factors, among others, so these acids possibly act as stimuli for this pathway. Activating the IKKβ complex that will
subsequently phosphorylate the IkBα proteins bound to NF-κβ, these will degrade, releasing NF-κβ. NF-κβ is subsequently translocated to the nucleus to promote the expression of genes that, in this case, could correspond to CCL5/RANTES. It is vital in future research to determine the expression of CCL5 in other muscles, both in the forelegs and hindlegs of animals, but mainly in the hindlegs, since these muscles are primarily affected by sarcopenia. In addition, it would be interesting to know if CCL5 expression is modified by positive factors for skeletal muscle, such as physical training.

11.5 Conclusion

Our results collectively suggest an increased ccl5 gene expression in the diaphragm muscle concomitantly with elevated serum bile acids and the development of sarcopenia.

Statements and Declarations

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Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Approval was granted by the ethics committee of the Universidad Andrés Bello in Santiago, Chile (Approval number 017/2020).

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Differential Fibrotic Response of Muscle Fibroblasts, Myoblasts, and Myotubes to Cholic and Deoxycholic Acids

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Abstract

Fibrosis is a condition characterized by an increase in the components of the extracellular matrix (ECM). In skeletal muscle, the cells that participate in the synthesis of ECM are fibroblasts, myoblasts, and myotubes. These cells respond to soluble factors that increase ECM. Fibrosis is a phenomenon that develops in conditions of chronic inflammation, extensive lesions, or chronic diseases. A pathological condition with muscle weakness and increased bile acids (BA) in the blood is cholestatic chronic liver diseases (CCLD). Skeletal muscle expresses the membrane receptor for BA called TGR5. To date, muscle fibrosis in CCLD has not been evaluated. This study aims to assess whether BA can induce a fibrotic condition in muscle fibroblasts, myoblasts, and myotubes. The cells were incubated with deoxycholic (DCA) and cholic (CA) acids, and fibronectin protein levels were evaluated by Western blot. In muscle fibroblasts, both DCA and CA induced an increase in fibronectin protein levels. The same response was found in fibroblasts when activating TGR5 with the specific receptor agonist (INT-777). Interestingly, DCA reduced fibronectin protein levels in both myoblasts and myotubes, while CA did not show any effect in these cell populations. These results suggest that DCA and CA can induce a fibrotic phenotype in muscle-derived fibroblasts. On the other hand, DCA decreased the fibronectin in myoblasts and myotubes, whereas CA did not show any effect in these cell populations. Our results show that BA has different effects depending on the cell population to be analyzed.
12.1 Introduction

Muscular fibrosis is an excessive increase in extracellular matrix (ECM) elements, which replace muscle tissue and negatively affect skeletal muscle function [1, 2]. The ECM components that increase in this condition are collagen type I and III and fibronectin [3]. Muscle fibers can synthesize ECM proteins, and it has even been shown to respond in vitro to a fibrotic stimulus, increasing fibronectin levels [4]. It has also been described that myoblasts, a cell population originating from satellite cells that can differentiate into muscle fibers, can increase collagen and fibronectin expression [5–7]. In addition, muscle-derived fibroblasts under fibrotic conditions increase the expression of ECM proteins such as collagens I and III, fibronectin, and metalloprotease inhibitors (TIMP). This latter inhibits the expression or activity of metalloproteinases (MMP), enzymes responsible for ECM degradation [8, 9]. Furthermore, fibroblasts proliferate under fibrotic conditions [10]. Together, these events favor ECM deposition and the development of fibrosis.

When the muscle is injured, one of the first participants are the endothelial cells, which are capable of releasing cytokines that will recruit leukocytes, in addition to releasing MMPs that will break down the basement membrane to facilitate immune cell infiltration into the muscle. These latter cells will fulfill the role of phagocytizing cell debris [1, 3]. Furthermore, satellite cells activate and proliferate, forming myoblasts that divide, fuse, and differentiate. Thus, new muscle fibers are formed, replacing damaged ones. Parallel to these processes, fibroblasts proliferate and secrete, together with other resident cells such as myoblasts and muscle fibers, a transient ECM that will function as a scaffold and facilitate the formation of new muscle fibers [3]. At this point is critical an acute and temporary inflammation for avoiding regeneration leads to fibrosis since chronic inflammation can cause the fibroblasts to resist apoptosis [8].

Fibrosis has been described in non-genetic chronic diseases that indirectly affect skeletal muscle, such as kidney disease, arterial hypertension, and type 2 diabetes [11]. Another chronic disease characterized by muscle weakness is cholestatic chronic liver diseases (CCLD). In these pathologies, a decrease in muscle mass has been described in animal models and humans. However, whether the skeletal muscle from CCLD presents fibrosis has not been evaluated [12, 13].

CCLD is a pathology characterized by progressive liver damage, eventually leading to cirrhosis. This last stage presents different alterations, such as liver fibrosis and hepatocellular dysfunction [14]. Therefore, it ultimately leads to a liver transplant. Of patients with cirrhosis, 40–80% have muscle weakness and develop sarcopenia, the loss of muscle mass,
strength, and function [15, 16]. This condition has profound implications for the transplant since people who present both sarcopenia and cirrhosis have lower post-transplant survival (40%), in contrast to people who do not show muscle weakness (80%) [13]. Besides, sarcopenia could help predict mortality in cirrhotic patients [17].

For studying the relationship between muscle weakness with CCLD, murine model sarcopenia derived from sclerosing cholangiopathy has recently been described for our laboratory [12]. This animal model develops liver damage ending in cirrhosis via induction fibrosis in the bile ducts [9]. Different features are described in this model, among them an increase in liver size [18] and a decrease in strength, muscle mass, and function [12]. Another feature in this model, which has not been published, is the increased fibronectin protein levels (data not shown). This antecedent, added to the loss of function previously described [12], allows us to infer that this tissue presents fibrosis in this model.

A typical characteristic of the murine model of sclerosing cholangiopathy-induced sarcopenia is the increased serum levels of bile acids (BA) [18, 19]. BA are amphipathic molecules produced in the liver from cholesterol and stored in the gallbladder [20]. They divide according to their origin into primary BA, the most abundant and synthesized in the liver, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), or secondary BA (or dehydroxylated derivates), which are primary BA modified by intestinal bacteria. These are the acids deoxycholic (DCA) and lithocholic (LCA) [20, 21]. The most abundant BA are CA, CDCA, and DCA [22].

The BA function has been classically associated with the fat emulsion to facilitate intestinal absorption. However, this role has been extended with its function as signaling molecules [21, 23, 24]. Since the 1990s, BA began to be described to act as hormones through nuclear receptors [25], the Farnesoid receptor (FXR). This receptor is a nuclear hormone receptor identified as N1H4. Farnesoid receptor expresses in tissues that participate in BA synthesis and absorption, such as the liver and the gut [26]. Another BA receptor is the Takeda-G-protein-receptor-5 (TGR5) membrane receptor, distributed in different tissues that do not necessarily participate in the BA synthesis pathways and are more associated with BA metabolic mechanisms [24]. In this context, TGR5 is expressed in the gallbladder, liver, adipose tissue, kidney, and skeletal muscle [27–29].

The metabolic role of TGR5 in skeletal muscle remains unclear, and some functions have been indirectly proposed to occur in muscle by data extrapolation from other tissues. Thus, TGR5 can induce the deiodinase 2 expression, which would increase energy expenditure and oxygen consumption, based on studies previously described for brown adipose tissue, in a mechanism dependent on CREB activated by PKA [27]. In addition, it has been proposed that TGR5 expression can be upregulated in skeletal muscle under exercise and could favor myogenesis [29]. However, its participation in pathologies that affect skeletal muscle has been poorly studied. In this line of evidence, recent reports from our laboratory indicate that the absence of the TGR5 expression prevents sarcopenia in mice with CCLD. This antecedent suggests that BA can mediate its effect on skeletal muscle through this receptor [30]. In addition, we also reported that DCA and CA induced atrophy in culture cells and skeletal muscle fibers, inducing an increment of the ubiquitin-proteasome system (UPS), oxidative stress, and mitochondrial dysfunction in a TGR5-dependent manner [30]. This evidence indicates that BA and TGR5 activation is detrimental to skeletal muscle tissue. However, its participation in fibrotic pathologies that affect skeletal muscle has not been studied.

Regarding fibrosis, the only antecedents that link this condition with the TGR5 receptor are described in renal tissue. In a diabetic nephropathy model, characterized by an increase in the expression of TGF-β and fibronectin, the TGR5 activation decreases the levels of fibrotic markers in glomerular mesangial cells [28, 31]. Therefore, activation of this receptor has a protective effect on renal fibrosis. However, the role of TGR5 in muscular fibrosis has not been determined.
Therefore, this work aims to evaluate the effect of CA and DCA on the fibrosis evidenced in the three cell populations derived from skeletal muscle: primary muscle fibroblasts, C2C12 myoblasts, and C2C12 myotubes.

### 12.2 Methods

**Animals**: C57BL/6J WT male mice (16 weeks old) were fed a standard diet. The animals (weighing 25–30 g) were kept in the mice housing of the Department of Biological Sciences of the Universidad Andrés Bello, at a controlled temperature (22–24 °C), with food and water ad-libitum and inverted 12-h light/dark cycles (Light: 8 P.M.–8 A.M.). Our experiments followed all international, national, and institutional suggestions and guidelines for animal care and use. Our studies and procedures with animals had the Animal Ethics Committee’s formal approval at the Universidad Andrés Bello (approval number 007/2016, March 2016).

**C2C12 cell culture**: C2C12 cell line was obtained from American Type Culture Cells (ATCC, CRL-1772) (Manassas, VA, USA). C2C12 is a sub-clone derived by Blau et al. [32] using the cell line established by Yaffe and Saxel from the hindlimb skeletal muscle of a wild-type C3H mouse [33]. Cells were maintained in a growth medium (DMEM supplemented with 10% fetal bovine serum (FBS)-1% antibiotic/antimycotic solution). Cells were trypsinized each time they reached 70% confluence. Myoblast maintenance was carried out in an incubator at 37 °C and 5% CO₂. Once the cells were grown to approximately 90% confluence, Myoblast maintenance was carried out in an incubator at 37 °C and 5% CO₂. Once the cells were grown to approximately 90% confluence, they were twice washed with HBSS and then supplemented with a differentiation medium (DMEM-2% horse serum (SC) and 1% antibiotic/antimycotic solution). This last medium was changed every 48 h to obtain cells fully differentiated at day 5. The maintenance of the myotubes during the differentiation was carried out in an incubator at 37 °C and 5% CO₂.

**Primary culture of fibroblasts**: C57BL6 mice were anesthetized with isoflurane until they were completely asleep and sacrificed by cervical dislocation. Once this was done, the muscles of the hindlimbs (tibialis anterior, soleus, extensor digitorum longus (EDL), and gastrocnemius) were removed. These muscles were placed in 35 mm plates with minimal medium (DMEM-1% antibiotic/antimycotic solution). The muscles were cleaned, removing fat and tendons. They were cut into small pieces and placed in 35 mm plates with freshly prepared growth medium (DMEM-10% FBS-1% antibiotic/antimycotic) for 10 days. During that time, they changed their medium periodically. Once this time had elapsed, the migration of cells from the muscle fragments was confirmed, the tissue was removed from the culture. The cells adhered to the plate were washed twice with PBS to remove muscle debris, and a growth medium (DMEM-10% FBS) was added and replaced every 2 days until they reached 50% confluence. Then, the cells were trypsinized and replated. Further 1 h, the supernatant was removed to enrich the cell population in cells with rapid adherence to the plate, mainly fibroblasts, to the detriment of other cell populations that could be present, mainly myoblasts [34]. After this step, the cells were cultured and amplified until obtaining enough cells for the experiments (not beyond passage 4). For experiments, fibroblasts were seeded at 70% confluence. Fibroblast maintenance was carried out in an incubator at 37 °C and 5% CO₂.

**Treatments with bile acids**: Cells (myoblasts, myotubes, and muscle-fibroblasts) were incubated with cholic acid (CA) (Sigma-Aldrich, St Louis, MO, USA) or deoxycholic acid (DCA) (Sigma-Aldrich, St. Louis, MO, USA) was added for the times indicated in each figure. The cultures designated as the control group (Vehicle) were incubated with DMEM. Once the incubations were finished, the plate was washed twice with 1 ml of cold PBS and frozen at −20 °C until protein extraction was carried out.

**Immunofluorescence microscopy**: The location of TCF4 and MyoD in the primary culture of fibroblast were analyzed by indirect immunofluorescence. Cells were fixed in 4% paraformaldehyde permeabilized with 0.05% Triton X-100 and incubated with rabbit
anti-TCF4 (1:200; Cell Signaling, Danvers, MA, USA) and rabbit anti-MyoD (1:50; Santa Cruz Biotech., Dallas, TX, USA) overnight. Alexa-Fluor 488 conjugated anti-rabbit (Thermo Fisher Scientific, Waltham, MA, USA) was used as the secondary antibody. For nuclear staining, the sections were incubated with 1 µg/ml Hoechst 33258 in phosphate-buffered saline (PBS) for 10 min. After rinsing, the cells were mounted with a fluorescent-mounting medium Fluoromount (Sigma-Aldrich, St. Louis, MO, USA) under glass coverslips. Images were captured using the Motic BA310 epifluorescence microscope (Motic, Hong Kong).

**Protein cell extracts:** Cells were washed 3 times with 2 ml cold PBS and then resuspended in RIPA lysis buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM Na2EDTA; 1 mM EGTA; 1% NP-40; 1% Sodium deoxycholate; phosphatase inhibitors: 1 mM orthovanadate; 1 mM NaF; 30 mM sodium pyrophosphate; protease inhibitors: 1 mM PMSF and 1 mM cocktail of protease inhibitors) 200 µl for myotubes and 100 µl for fibroblasts and myoblasts, and were subsequently incubated on ice for 15 min. Finally, the lysate was centrifuged at 10,000 × g for 10 min at 4 °C, the supernatant was recovered, and the pellet, corresponding to cell debris, was discarded. Protein samples were stored at −20 °C for total protein measurement and subsequent use in Western blot assays. The bicinchoninic acid method (microBCA protein assay kit) (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the total protein concentration. The reaction was performed in 96-well plates according to the manufacturer’s instructions. The OD of 575 nm was measured on the plate reader. The absorbance values obtained were corrected for the respective blanks and interpolated with a calibration curve made from bovine serum albumin (BSA) solution of known concentration.

**Western blot analysis:** For Western blot analysis, 8 or 30 µg of protein were used for fibronectin and TGR5, respectively. Protein samples were mixed with loading buffer (60 mM Tris-HCl pH 6.8; 25% glycerol; 2% SDS; 0.7 M β-mercaptoethanol; 0.1% bromophenol blue) in a 1:5 volume to load. Electrophoresis was then performed under denaturing conditions on 8% or 12% polyacrylamide gels (SDS-PAGE) with running buffer (25 mM Tris-HCl pH 8.3; 192 mM glycine; 0.1% w/v SDS) at 100 V for 3 h. Subsequently, the proteins were transferred to a PVDF membrane (Thermo Fisher Scientific, Waltham, MA, USA), previously activated with methanol for 5 min, using a transfer buffer (25 mM Tris-HCl pH 8.3; 192 mM glycine; 20% v/v methanol) and subjected to 400 mA for 150 min at room temperature. After transfer, the membrane was incubated in methanol for 5 min and allowed to dry at room temperature. Next, the membrane was blocked with a solution of TBS (150 mM NaCl; 50 mM Tris-HCl pH 7.5) with 0.1% Tween 20 and 5% BSA (TBS-Tween 20-BSA) for 1 h. At the end of this time, the membrane was incubated with primary antibodies (anti-fibronectin, -β-actin, -TGR5, -TCF4, and -MyoD) diluted in a blocking solution at 4 °C overnight. The next day, the membrane was washed 3 times with TBS-0.1% Tween 20 for 5 min and then incubated with the secondary antibody diluted in TBS-Tween 20-BSA for 1 h at room temperature. Subsequently, the membrane was washed 3 times with TBS-0.1% Tween 20, and 3 times with PBS. The binding of the primary/secondary antibody complex to the proteins of interest was revealed by chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA), detected through an image documentation system FOTO/Analyst Luminary/FX, Fotodyne (ThermoFisher Scientific, St. Waltham, MA, USA). The quantification of the bands obtained was carried out using the Image J program (NIH, Bethesda, MD, USA). The protein levels were normalized related to the levels of β-actin, used as a loading control.

**Statistical analysis:** The statistical analysis of the data was performed with Prism 9.0 analysis software (GraphPad Software, San Diego, CA, USA). The normality of the data was determined with the Shapiro-Wilk test. Normal data were analyzed with a t-test to compare the two groups. One-way ANOVA with Tukey multiple
comparison tests was used to analyze three or more groups. Differences were considered significant when the $p$-value was $< 0.05$.

### 12.3 Results

**Skeletal muscle-derived fibroblasts express bile acid receptor TGR5**

Fibroblasts are a cell population that encompasses different cells that may differ from each other depending on the tissue in which they are found [35]. This antecedent suggests that these cells must be extracted from this tissue to evaluate the effects on muscle fibroblasts. The procedure and characterization of primary cultures of fibroblasts obtained from the muscles of the hindlimbs of mice have been previously described [36, 37]. The skeletal muscle fibroblast marker TCF4 [38] was used to evaluate the presence of fibroblasts. As observed in the graph of Fig. S1, skeletal muscle-derived fibroblasts reach a 95% purity relative to myoblasts detected by the MyoD marker.

Since TGR5 is the only receptor for bile acids described in muscle tissue [27], the presence of this receptor was explicitly evaluated in the population of muscle fibroblasts. As can be seen in Fig. S2, the TGR5 receptor is expressed in muscle-derived fibroblasts. This result indicates that fibroblasts are sensitive to BAs, and TGR5 could mediate its effects.

**Deoxycholic and cholic acids increase fibronectin levels in the primary culture of muscle fibroblasts**

Once the fibroblasts were obtained and their identity and purity verified, they were treated with DCA at different concentrations to evaluate their effect on fibronectin protein levels. The fibroblasts were treated for 48 h with DCA in several concentrations previously tested for not affecting cell viability (from 30 to 180 µM) [30]. In addition, TGF-β was used as a positive control since it has been reported to induce an increase in fibronectin in muscle cells, including fibroblasts [34]. As shown in Fig. 12.1a, the fibroblasts are sensitive to DCA and TGF-β, increasing fibronectin levels. Figure 12.1b shows the quantification evidenced by an increase in the fibronectin levels under all the concentrations of DCA used, reaching double the values of the fibrotic marker in all of them, independent of the concentration. Therefore, it can be concluded that DCA induces an increase in the fibrotic-like phenotype in primary mouse skeletal muscle fibroblasts.

In muscle-derived fibroblasts, the effect of CA on fibronectin protein levels was also evaluated by incubating them at increasing concentrations ranging from 50 to 500 µM (Fig. 12.2a). These concentrations were previously shown not to alter cell viability assessed in muscle cells [30]. Figure 12.2b corresponds to the quantification of Fig. 12.1a, showing an increase in the fibronectin protein levels at the highest CA concentrations (250 and 500 µM), reaching approximately twice the baseline values. Therefore, it can be concluded that CA may be an inducer of fibrosis in muscle fibroblasts.

Since DCA and CA are ligands for the TGR5 receptor and muscle-derived fibroblasts express this receptor, we evaluated the fibrotic effect of TGR5 activation with a specific agonist, INT-777. Figure S3a shows the fibronectin levels in response to INT-777 at 10 and 25 mM. The quantification of the data indicates that TGR5 activation mediated by INT-777 increments the fibronectin protein levels (Fig. S3b).

In short, when incubated with DCA, CA, or a specific TGR5 agonist, the muscle-derived fibroblasts respond by increasing fibronectin levels. This effect occurs at all DCA concentrations and only at the highest CA concentrations, suggesting that the receptor that mediates this effect is more sensitive to DCA than to CA, coinciding with the TGR5 receptor affinities of activation. These results indicate that the TGR5 receptor could mediate the BA-induced fibrotic effect on muscle-derived fibroblasts.

**Deoxycholic acid, but not cholic acid, reduces basal protein levels of fibronectin in C2C12 myoblasts**

We evaluated the effect of the DCA and CA on fibrosis in myoblasts, another mononuclear cell
population that participates in muscle fibrosis [7]. C2C12 myoblasts were incubated for 48 h with DCA or CA at the same doses as in precedent experiments. Figure 12.3a shows the Western blot for fibronectin of myoblasts incubated with several concentrations of DCA. The quantification shown in Fig. 12.3b indicates that myoblasts reduce the fibronectin levels compared to the vehicle in all DCA doses.

On the other hand, when myoblasts are incubated with CA, and the fibronectin levels are evaluated by Western blot, as seen in Fig. 12.4a, it is possible to observe no changes compared to vehicle conditions (Fig. 12.4b).

We observed that C2C12 myoblasts are sensitive to DCA but not CA, decreasing the basal fibronectin levels, contrary to the evidence observed on muscle-derived fibroblasts.

**Deoxycholic acid reduces the basal protein levels of fibronectin in C2C12 myotubes, while CA does not modify them**

Myotubes are in vitro models of muscle fibers described to participate in muscle fibrosis [4]. Thus, myotubes were incubated for 48 h with DCA. Figure 12.5a shows a representative Western blot in myotubes showing TGF-β as the positive control. The quantification shown in Fig. 12.5b indicates that DCA produces a 50% reduction in the basal protein levels of fibronectin, like the effect observed in myoblasts. However, unlike myoblasts, this response can only be seen at the highest DCA concentrations (120 and 180 µM), suggesting that myotubes are less sensitive than myoblasts. TGF-β did not
produce the increase in fibronectin that could be observed in the other cell types, which is consistent with previous reports [39] that explain the lesser sensitivity of myotubes to TGF-β signaling than myoblasts.

On the other hand, but in the same line that results shown in myoblasts, CA does not generate changes in the protein levels of the fibronectin, as can be seen in the Western Blot (Fig. 12.6a) and its respective quantification (Fig. 12.6b).

Together, these data conclude that only DCA produces a response in the myotubes and that this would decrease the fibronectin protein levels.

### 12.4 Discussion

In the present study, we have observed that muscle-derived fibroblasts increment the fibronectin protein levels in response to DCA and CA. On the other hand, myoblasts and myotubes present decreased fibronectin levels under DCA incubation, whereas both cell populations are irresponsive to CA.

Increased serum BA are characteristic of CCLD, a disease highly relevant in the Chilean population. According to the department of statistics and health information (DEIS), the mortality rate related to liver diseases and intrahepatic bile ducts corresponds to 5.56% of all the causes of death within the period 2000–2011 (DEIS-MINSAL).

The role of skeletal muscle during CCDL has begun to gain relevance since the correlation between muscle weakness and lower post-liver transplant survival was described [13]. Also, it has been demonstrated in a murine model that CCDL can induce sarcopenia characterized by decreasing muscle strength, locomotion functionality, reduction in fiber diameter, decreased MHC
protein levels, and increased UPS markers [12]. The effect of CCDL on skeletal muscle depends on an increase in ROS levels and the presence of the TGR5 receptor [19, 40]. Besides, an increase in a marker of fibrosis, fibronectin, has been found in skeletal muscle (data not shown), which correlated with a decrease in contractile function [12]. DCA and CA can replicate all sarcopenic effects in cell culture and skeletal muscle fibers [30]. In this line, this is the first report of possible fibrotic effects of BA in skeletal muscle.

The antecedents of the BA role in skeletal muscle have focused on sarcopenia. This condition is defined as decreased muscle mass, strength, and physical function [41]. Therefore, in recent years exercise has been proposed as a therapy to mitigate the symptoms of this disease [42]. However, fibrosis in the skeletal muscle could prevent optimal muscle mass recovery because the functional muscle tissue is replaced by fibrotic tissue [43]. In addition, fibrosis can generate a microenvironment that prevents muscle repair and could reduce the efficiency of exercise-related therapy.

In the model of cholestatic disease implemented for our group [18], an increase in a marker of fibrosis, fibronectin, has been found in skeletal muscle (data not shown), which correlated with a decrease in the contractile function [12]. These antecedents adjust to the definition of fibrosis. In addition, in previous work from our group, it was described that BA could act as soluble factors for inducing sarcopenia in a murine model and induce a sarcopenic-like phenotype in vitro, which depended on the TGR5 expression [30].

In the present study, three cell populations participating in skeletal muscle fibrosis, such as fibroblasts, myoblasts, and myotubes, were evaluated regarding their fibrotic response to DCA and CA by assessing the fibronectin protein levels, a typical fibrotic marker.
Our results show that skeletal muscle-derived fibroblasts expressed the TGR5 receptor. The presence of TGR5 in a related cell type was described in the cell line 3T3-L1 [44], which is used as a precursor of adipocytes. This research explains that the activation of TGR5 in adipocyte precursor cells favors differentiation into brown adipocytes. Although this work did not explicitly evaluate the presence of the receptor, 3T3 cells respond to the incubation with the specific TGR5 agonist INT-777, which strongly suggests the presence of the receptor. Therefore, our work is the first study that describes the presence of TGR5 in muscle-derived fibroblasts.

Muscle-derived fibroblasts respond by increasing fibronectin protein levels when they are incubated with DCA and CA, as well as with INT 777, suggesting the TGR5 activation and the participation of this receptor in the fibrotic response to DCA and CA. The behavior of muscle-derived fibroblasts in response to BA supports the idea that TGR5 could be participating in the influence of CA and DCA on fibroblasts. In this line, the response to DCA is observed even at the lowest concentrations evaluated (30 and 60 µM), while for CA, the response was seen at the highest concentrations (250 and 500 µM). These results are consistent with the TGR5 affinity for these BA since TGR5 has a higher affinity for DCA than CA [45]. In addition, CA is an agonist for TGR5 and not for FXR, reinforcing the idea that TGR5 mediates the CA effects [23]. However, to fully confirm the participation of TGR5 in the increase of fibronectin levels in fibroblasts, complementary studies in fibroblasts without the receptor, decreasing its expression, or using a TGR5 antagonist must be performed.

Fig. 12.4 Cholic acid does not change basal protein levels of fibronectin in C2C12 myoblasts. Myoblasts were incubated with 50, 100, 250, and 500 µM of cholic acid (CA) for 48 h. **a** Protein levels of fibronectin were detected by western blot analysis using b-actin levels as a loading control. TGF-β was used as a positive control (+). Molecular weight markers are depicted in kDa. **b** The quantitative analysis of value is expressed as a fold of change. Values correspond to the mean ± SEM (n = 3, *p < 0.05 vs. control condition (without CA); one-way ANOVA and Tukey’s multiple comparison test).
molecular mechanisms involved in increased fibronectin levels. In this sense, an imbalance in the proteostasis of fibronectin can be explained by increased protein synthesis and/or decreased degradation. If we consider that the canonical signaling pathway of TGR5 (G-protein and cAMP increase) could mediate the imbalance in proteostasis of fibronectin, it could be speculated that an increase in fibronectin levels is produced by increased synthesis, similar to the finding described in mesangial cells that link the PKA-CRE pathway [46]. Another mechanism that could explain the increase in fibronectin synthesis is the DCA- and CA-dependent activation of AP-1 via the ERK1/2 pathway. It has been described that BA, via TGR5, can activate ERK 1/2 in different cell types, such as macrophages, colonocytes, and cholangiocytes [47–49]. Besides, the ERK 1/2 phosphorylation can induce the expression of fibronectin in decidual fibroblasts by activating AP-1 [50]. In addition, it has been described that ERK1/2 participates in skeletal muscle fibrosis [4]. Therefore, experiments should be conducted to evaluate the possible role of ERK1/2 in the increase of fibronectin levels induced by BA in muscle fibroblasts.

Two mechanisms have been described for fibronectin degradation: one dependent on extracellular proteases such as MMPs [51] and another dependent on internalization and subsequent binding to lysosomes [52]. In the former, it has been shown that muscle cells can express different MMPs, such as MMP-1, MMP-2, MMP-3, MMP-9, and MMP-14, capable of degrading fibronectin [51, 53]. In colorectal cells, DCA increases the expression of MMP-9 [54, 55]. This antecedent is relevant for myoblasts, a cell type that expresses MMP-9 [56], since it could explain the decline in the fibronectin levels when myoblasts are exposed to DCA.

MMP-14 has been shown to participate in the mechanism of fibronectin degradation dependent...
on endocytosis. Thus, cells lacking MMP-14 reduce fibronectin endocytosis [57]. MMP-14 has been proposed to have an anti-fibrotic role in skeletal muscle since, in addition to degrading fibronectin, it can activate MMP-2, another enzyme that participates in fibronectin degradation [58]. Further studies must be performed to elucidate the possible role of MMP-14 in the fibrotic response of muscle-derived fibroblasts, myoblasts, and myotubes.

In myoblasts and myotubes, no difference was found in fibronectin levels when treated with CA. In the case of DCA treatments, an opposite response to fibroblasts was seen since DCA decreases fibronectin protein levels. This result agrees with the antecedent showing that TGR5 activation reduces the protein levels of fibrotic markers, such as fibronectin, in kidney cells [28].

It has recently been described that BAs can stimulate other receptors besides TGR5 and FXR. Among these are S1P and muscarinic receptors [59]. Even though skeletal muscle express 4 of the 5 isoforms (S1P1, S1P2, S1P3, and S1P4) [60], BA only activates S1P2 [61]. Although initially, it was shown that conjugated BA only achieved this activation, then it was described that DCA could activate the S1P2 receptor in macrophages [62]. S1P2 receptor declines its expression during skeletal muscle cells differentiation [63] process in which there is an increase in MMP-2 and -14 and fibronectin degradation [58]. Thus, it is possible to speculate a decrease in fibronectin levels if DCA activates the S1P2 receptor. Interestingly, this receptor is decreased in myotubes doing more sensitive the myoblasts to its effects. This antecedent

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**Fig. 12.6** Cholic acid does not change basal protein levels of fibronectin in C2C12 myotubes. Myotubes were incubated with 50, 100, 250, and 550 µM of CA for 48 h. **a** Protein levels of fibronectin were detected by western blot analysis using β-actin levels as a loading control. TGF-β was used as a positive control (+). Molecular weight markers are depicted in kDa. **b** The quantitative analysis of value is expressed as a fold of change. Values correspond to the mean ± SEM (n = 3, *p < 0.05 vs. control condition (without CA); one-way ANOVA and Tukey’s multiple comparison test).
correlates with the reduced response to DCA shown by the myotubes. This antecedent proposes that the S1P2 receptor could mediate the effect of DCA on myoblasts and myotubes.

Muscarinic receptors are G-coupled receptors for acetylcholine, with five subtypes described. M2 and M4 receptors have been seen to have Gi activity, that is, their activation inhibits the enzyme adenylate cyclase. While M1, M3, and M5 have a Gq activity. There are no muscarinic receptors in adult skeletal muscle, only nicotinic receptors. However, the presence of these receptors has been seen in cell cultures derived from skeletal muscle and when differentiating. Experiments suggest only the presence of M1 and M3 in these cells [64]. The interaction between BA and these receptors has only been described for conjugated BA, with DCA modulating M2 and M3 receptors [65, 66]. It has been described that the M3 receptor absence reduces collagen I and fibronectin in lung tissue [67]. The latter is consistent with results found in myotubes and myoblasts in the present study since DCA could act as an antagonist of the M3 receptor and reduce the expression of fibronectin.

Once the effects of BA on muscle fibroblasts, myoblasts, and myotubes have been defined, it would be interesting to evaluate if one cell type influences the other. It has been described that these cells can communicate between themselves and modify their functions and protein expression. For example, Fry in 2016 showed that myoblast-isolated exosomes from exercised mice reduce the expression of ECM components via miRNA 206 in fibroblast cultures [68]. Similarly, fibroblasts derived from people with Duchenne muscular dystrophy, a disease characterized by fibrosis and skeletal muscle damage, release exosomes that, when added to cell cultures, favor the synthesis of ECM proteins [68]. On the other hand, it has been suggested that fibroblasts help with efficient myogenesis, as co-cultures of fibroblasts and myoblasts have shown [69]. Therefore, the communication between these three cell types could alter the effects of the BA described in this work since they were evaluated individually here. Eventually, the interaction between these cell populations could converge in the presence of fibrosis in mice with CCLD, despite myotubes and myoblasts showing a decrease in the fibrotic marker when treated with DCA.

12.5 Conclusion
In summary, these results suggest that the bile acids DCA and CA can induce skeletal muscle fibrosis through a differential response to muscle fibroblasts through the TGR5 receptor. The effect of DCA in myoblasts and myotubes was to decrease the fibronectin levels, whereas CA did not change the levels of this fibrotic protein. Further studies must be conducted to quantify each cell type’s actual and total contribution to the skeletal muscle and how it can induce muscle fibrosis in the murine model of CCLD.

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Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Approval was granted by the ethics committee of the Universidad Andrés Bello in Santiago, Chile (Approval number 017/2020).

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BMAL1 Regulates Glucokinase Expression Through E-Box Elements In Vitro

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Abstract

The organization of a circadian system includes an endogenous pacemaker system, input pathways for environmental synchronizing (entraining) stimuli, and output pathways through which the clock regulates physiological and behavioral processes, for example, the glucose-sensing mechanism in the liver. The liver is the central regulator of metabolism and one of our peripherals clocks. In mammals, central to this pacemaker are the transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK) and BMAL1 (Brain and Muscle ARNT-Like 1). BMAL1 dimerizes with CLOCK, and this heterodimer then binds to the E-box promoter elements (CACGTG) present in clock and clock-controlled genes (CCGs). However, we are just beginning to understand how output pathways and regulatory mechanisms of CCGs are involved in rhythmic physiological processes. Glucokinase (GCK) is a fundamental enzyme in glucose homeostasis, catalyzing the high Km phosphorylation of glucose and allowing its storage. Moreover, gck is a dependent circadian gene. This study aims to determine the contribution of clock genes to hepatic gck expression and to define the specific role of E-box sequences on the circadian regulation of hepatic gck. Results showed that gck expression follows a circadian rhythm in rat hepatocytes in vitro. Accordingly, bmal1 expression induces the glucokinase circadian rhythmic expression in hepatocytes and the analysis of human and rat gck promoters, indicating the presence of E-box regions. Moreover, the basal activity of gck promoter was increased by clock/bmal1 co-transfection but inhibited by Period1/Period2 (per1/per2).
Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| bHLH-PAS     | Basic helix–loop–helix/per-ARNT-SIM |
| bHLHz        | Basic region/helix–loop–helix/leucine zipper |
| BMAL1        | Brain and muscle ARNT-like 1 |
| CLOCK        | Circadian locomotor output cycles kaput |
| CRY          | Cryptochrome |
| DMEM         | Dulbecco’s modified Eagle’s medium |
| EGTA         | Ethylene glycol-bis-(2-aminoethyl ether) N,N,N’,N’-tetraacetic acid |
| FBS          | Fetal bovine serum |
| GCK          | Glucokinase |
| LGCK         | GCK liver |
| GLUT2        | Glucose transporter 2 |
| GFP          | Green fluorescent protein |
| hGK          | Human GK (cloning) |
| HEPES        | N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid |
| MMH-D3       | Met murine hepatocytes |
| PER1         | Period 1 |
| PER2         | Period 2 |
| RT-qPCR      | Quantitative reverse transcription PCR |
| SCN          | Suprachiasmatic nucleus |

Keywords

Hepatocytes · Glucokinase · BMAL1 · E-box

13.1 Introduction

In most living organisms, from bacteria to humans, physiological and behavioral processes are coordinated by circadian clocks. The circadian rhythm helps organisms adjust and anticipate the daily environmental changes, which is well represented by the daily sleep and wakefulness pattern, allowing synchronization of mammals’ physiology to the 24-h solar cycle. Such rhythmic oscillations are thought to have evolved in response to the daily light/dark rhythms [1]. Moreover, the internal clocks regulate daily eating, blood pressure, and hormone regulation patterns, which are critical for mammal functions and activities [2–4]. Much is known about the molecular and cellular mechanisms that govern the circadian clock; by contrast, much less is known about how the activity of circadian clocks causes physiological outputs to be rhythmic. Remarkably, the circadian system comprises two types of clocks set through daily feeding: the central pacemaker and the peripheral clock. The central pacemaker clock in the suprachiasmatic nucleus (SCN) is set by light and can synchronize peripheral clocks of other tissues through systemic signals. However, the signals that regulate these clocks and the molecular mechanisms in different types of cells are less known. In addition, other stimuli different from light can also regulate peripheral oscillators and dissociate rhythms of the peripheral clock from those of the SCN. Thus, for instance, in mice [5] and rats [6], the clock in the liver can be entrained by daily feeding regimes that do not alter the phase of the SCN clock. Thus, suggesting that each circadian output depends in vivo on complex coordination between the activity of the SCN and peripheral clocks. Meanwhile, the peripheral Circadian clocks, such as those found in the liver, are regulated by various signals, including body temperature, hormone metabolites, and feeding/fasting cycles influenced by food availability. Although the SCN is a synchronizer of peripheral clocks, peripheral clocks can be uncoupled from its central control. Through changes in the feeding schedule, the phase relationship between the central clock in the SCN and the clocks in the liver can be altered [5], suggesting that changes in metabolism caused by alterations in feeding rhythm may affect the circadian system. Specifically, the expression of some enzymes and transporter can be affected by clock genes and in a complementary way, the enzymes of the metabolism and proteins that regulate transcription can influence the central
core of the clock. There is evidence in animal models of the relationship between the disruption of clock genes, the loss of rhythmicity, and changes in metabolic homeostasis [5, 6].

Hepatic function, including glucose and lipids metabolism, is fine-tuning and controlled by a rhythmic regulation as circadian profiles of critical enzymes and transporters have been demonstrated in mammals’ livers [3, 7–9]. Several organs regulate metabolism, however, the liver is directly related to glucose metabolism. In this context, the circadian output pathways are a reflection of the physiological state and therefore the liver cells are capable of sensing and responding adaptively to changes in the environment to modify processes of entry and exit of nutrients. The SCN pacemaker oscillator is very sensitive to light signals, however, in the liver, the circadian clock is largely insensitive to the light regime, but its phase and amplitude are influenced by feeding activity [5, 6]. The liver oscillator is thought to help the organism adapt to a daily pattern of food availability by temporally tuning the expression of many genes regulating metabolism and physiology. Such temporal regulation of metabolism is important since the absence of a robust circadian clock predisposes the organism to various metabolic dysfunction and diseases [10]. Hepatocyte cultures have been used for analyzing circadian gene expression. Through bioinformatics analysis it was found 1130 circadian mRNA in Met Murine Hepatocytes (MMH-D3). This is an in vitro culture cells model, which is isolated from the central clock, showing an autonomous clock that could regulate many genes with circadian rhythm [11]. However, more studies are needed to understand the connection of the liver clock with the central clock and its real autonomy.

The liver is essential in buffering circulating glucose daily [7]. The liver’s circadian circuit adaptively assimilates nutrients such as glucose through glycolysis or glycogen synthesis [12, 13]. In particular, maintaining glucose homeostasis is one of the critical physiological functions of the liver. Glucose uptake into the hepatocyte, gluconeogenesis, and glycogenolysis must be sustained over daily feeding and fasting periods [1]. In this sense, hexokinase IV, also called glucokinase (GCK), is a crucial glycolysis and glycogen synthesis enzyme. It is associated with significant diseases, such as diabetes and obesity [14]. Hexokinase IV/GCK plays a fundamental role in glucose homeostasis. Glucose is transported into the hepatic cells by glucose transporter 2 (GLUT2), Glucokinase, with low affinity (Km 10–15 mM), phosphorylate glucose to produce glucose-6-phosphate and allow its storage via the glycogen synthesis pathways [15]. The product, the glucose-6-phosphate, does not inhibit GCK. The role of GCK is fundamental both in the liver and the pancreas. Knockout mice that do not have this enzyme in the beta cell of the pancreas die within three days of birth from acute hyperglycemia [16, 17]. In normoglycemia, GCK is detected in both nuclei and cytoplasm of parenchymal cells, and its subcellular localization seems to play on GCK function [18]. The regulation of this enzyme is fine and complex. Because enzyme localization is critical to its function, GCK regulatory protein (GKRP) can phosphorylate the enzyme to modulate GCK activity and mediate its nuclear translocation. [19, 20]. The circadian clock regulates the expression of certain key metabolic enzymes and transport systems, like GLUT2 and GCK. So, it is necessary to understand better the molecular mechanisms through which the circadian clock will regulate hepatic GCK expression.

Many studies support that insulin is a principal regulator of lgck gene expression. The transcriptional regulation of gck in the liver is widely studied, and several regulatory sites along its promoter region have been isolated (reviewed in [21, 22]). Among the regulatory sites described for this enzyme, the E-box sequence seems to play a critical role in the transcriptional activation of the gck promoter gene [23]. Namely, as part of the circadian clock mechanism, the E-box sequences are located within the promoter region of several clock genes and are critical for recruiting other clock elements to produce proper circadian rhythmicity [24, 25]. The internal mechanism of the clock involves a series of proteins/transcription factors that self-regulate in the form of interlocked transcriptional negative
feedback loops: Circadian Locomotor Output Cycles Kaput (CLOCK), and Brain and Muscle ARNT-Like 1 (BMAL1); Period (per) 1 and 2 and Cryptochrome (cry). For instance, the basic helix–loop–helix/Per-ARNT-SIM (bHLH-PAS)-containing transcription factors, CLOCK, BMAL1 form heterodimers that bind to the E-box enhancer elements in the promoters of the per 1 and 2 and cry genes, which increases their transcription. Furthermore, PER and CRY inhibit the BMAL1-CLOCK complex, which inhibits their expression, along with PER and CRY degradation, allowing the initiation of a new circadian cycle [26, 27]. The heterodimer BMAL1-CLOCK binds to the E-box promoter elements (CACGTG) present in clock and clock-controlled genes (CCGs) for example, gck. However, we are just beginning to understand how output pathways and regulatory mechanisms of CCGs are involved in rhythmic physiological processes.

The gene gck regulatory mechanisms have been studied; however, few studies have involved the circadian clock as part of its regulation. In the liver promoter of the gck gene, there is a cis-acting element of the E-box denominated P2 that is functional and active. This element, P2, is localized from nt-89 to -81 in relation to the transcriptional starting site in the gck rat gene. P2 has a canonical sequence (CACGTG) that binding factors of the basic region/helix–loop–helix/leucine zipper (bHLH zipper), which could suggest its regulation by the central core of the clock [7, 28–30]. But, despite the presence of functional cis-acting elements in the gck promoter, the precise function of these regions or their relationship to clock genes has not been elucidated so far.

This study aims to determine the contribution of clock genes to hepatic gck expression and to define the specific role of E-box sequences on the circadian regulation of hepatic gck promoter.

Results showed that gck expression follows a circadian rhythm in rat hepatocytes in vitro. Accordingly, Bmal1 induces the glucokinase circadian rhythmic expression in hepatocytes and the analysis of human and rat glucokinase promoters, indicating the presence of E-box regions. Moreover, the basal activity of the gck promoter was increased by clock/bmal1 co-transfection but inhibited by per1 and per2 co-transfection. Thus, the data suggest that the clock proteins tightly regulate the transcriptional activity of the gck promoter.

13.2 Methods

General nomenclature guideline: For the gene/protein nomenclature it was used the guideline recommended by Oxford Academy.

Animal housing conditions: All animals were handled according to Animal Welfare Assurance. All animal work was carried out following the ARRIVE guidelines (https://arriveguidelines.org, accessed July 1, 2021). Female adult Sprague-Dawley rats weighing 250 g were used in all experiments. Animals were housed in a separate animal room with constant temperature (21 ± 2 °C) and a controlled 12-h light/12-h dark cycle; lights were turned on every day at 7:00 a.m. Animals had free access to a standard rodent diet (Lab Diet, 5P00 Prolab RMH 3000, Purina Mills, St. Louis, MO) and tap water.

Hepatocyte isolation and culture: Hepatocytes were isolated from Sprague-Dawley overnight-fasted rats (~250 g) using the collagenase-perfusion of the liver through the portal vein, essentially [31]. Rats were anesthetized by intraperitoneal injection of ketamine:xylazine:ingor (2:2:1). The portal vein was cannulated with an 18-gauge intravenous catheter. The liver was perfused with a calcium-free buffer solution containing 130 mM NaCl, 3 mM KCl, 1 mM NaH2PO4, D-glucose 10 mM, 0.5 mM ethylene glycol-bis-(2-aminoethyl ether) N, N', N'-tetraacetic acid (EGTA), and 10 mM N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES) (pH 7.4) maintained at 37 °C, at a rate of 10 ml/min for 10 min. This was followed by perfusion with a perfusion buffer solution containing 130 mM NaCl, 3 mM KCl, 1 mM NaH2PO4, D-glucose 10 mM, 0.5 mM ethylene glycol-bis-(2-aminoethyl ether) N, N', N'-tetraacetic acid (EGTA), and 10 mM N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES) (pH 7.4) maintained at 37 °C, at a rate of 10 ml/min for 10 min. This was followed by perfusion with a perfusion buffer solution containing 4 mM CaCl2 and 0.04% p/v collagenase Type IV (Sigma-Aldrich, St. Louis, MO, USA) for 5 min (10 ml/min). Flow rates and
pressures were controlled by a perfusion pump (Masterflex Variable Speed Peristaltic Pump Model 7520-00). The liver was transferred to 10 mm dishes with 25 ml perfusion buffer solution and was mechanically dissociated into single cells. Hepatocytes were washed in perfusion buffer solution and suspended in Dulbecco’s modified Eagle’s medium (DMEM) with 5.5 mmol glucose, 10% fetal bovine serum (FBS) (Thermo Scientific, Waltham, MA) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 ng/ml insulin, 100 nmol/L dexamethasone and 2.5 mg/ml fungizone (Thermo Scientific). Cells were centrifuged at 100 × 2g for 2 min at 4 °C and resuspended in DMEM 10% FBS with supplements. Cell viability was determined by Trypan blue staining and was > 90%. Hepatocytes were then plated into collagen type I (Gibco, NY, USA)-coated six well-plates at a density of 1.0 × 10^6 cells/dish. The medium was replaced 2 h after plating.

Cell cultures and transfections: Rat hepatocyte primary cultures were transfected with commercial plasmid SureSilencing shRNA with a pshBMAL1/amp containing a green fluorescent protein (GFP) expression cassette and scrambled vector (Qiagen, Valencia, CA, USA), using lipofectamine 2000 (Invitrogen). Transfection was conducted in the presence of OptiMEM (Gibco) supplemented with 10% FBS (Thermo Fisher Scientific) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 ng/ml insulin, 100 nmol/L dexamethasone and 2.5 mg/ml fungizone (Thermo Scientific). Cells were centrifuged at 100 × g for 2 min at 4 °C and resuspended in DMEM 10% FBS with supplements. Cell viability was determined by Trypan blue staining and was > 90%. Hepatocytes were then plated into collagen type I (Gibco, NY, USA)-coated six well-plates at a density of 1.0 × 10^6 cells/dish. The medium was replaced 2 h after plating.

The human hepatoma cell line Hep3B was cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 25 mM glucose, supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% fetal bovine serum (FBS, Invitrogen) in 60 cm^2 Tissue culture flasks (Corning, USA) and maintained in an incubator at 37 °C at 95% humidity and 5% CO_2. When the dishes reached 80% confluently, the cells were trypsin-detached and culture-expanded in a fresh medium. 

ImageJ software analyses: Fluorescence images were analyzed using ImageJ software (version 1.52a, included with Java 1.8.0_112, National Institutes of Health, USA). The raw fluorescence images were analyzed to select the positive GFP cells. The positive cells were counted and compared in both conditions to measure the efficiency of transfection.

Measurement of mRNA from primary hepatocyte cultures: Quantitative reverse transcription PCR (RT-qPCR) analysis was used to measure the expression of the hepatocyte cyclophilin, gck, clock, bmal1 and per2. The following sets of primers were used: cyclophilin, sense 5′-ATA ATG GCA CTG GTG GCA AGT C-3′ and antisense 5′-ATT CCT GGA CCC AAA ACG CTC C-3′; gck, sense 5′-GTG AGG TCG GCA TGA TTG T-3′ and antisense 5′-TCC ACC AGC TCC ACA TTC T-3′; clock, sense 5′-GCG GCA GAA TAG CAC CCA GAG T-3′ and antisense 5′-ACT TGG CAC CAT GAC GGC CC-3′; bmal1, sense 5′-CCG TGG ACC AAG GTA GA-3′ and antisense 5′-CTG TGA GCT GTG GGA AGG TT-3′; per2, sense 5′-ACG CTG GCA ACC TTG AAG TA-3′ and antisense 5′-GAC ACA GCC ACA GCA AAC AT-3. Total RNA was isolated from the primary hepatocyte cultures using Trizol (Invitrogen). For RT-PCR, 1 µg of RNA was incubated in a 10 µl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl_2, 20 U RNase inhibitor, 1 mM dNTPs, 2.5 µM oligo d (T) primers, and 50 units of MuLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA) for 10 min at 23 °C followed by 30 min at 42 °C and 5 min at 94 °C. Parallel reactions were performed without reverse transcriptase to control the presence of contaminant DNA. PCR reactions were carried out in an Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA). RT-qPCR was performed using the qPCR Master Mix kit for Brilliant II SYBR Green (Agilent Technologies, Inc.) in a final volume of 12.5 µl consisting of 1x SYBR Green Master Mix, 500 nM of each
primer, and 1 µl of cDNA sample. All reactions were performed with an initial denaturation of 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, annealing for 30 s at 60 °C, and extension for 1 min at 72 °C. The relative expression of gck and clock genes to cyclophilin mRNA was calculated based on the PCR efficiency method.

Cloning and site-directed mutagenesis of the gck promoter: The genomic sequence encoding the liver human gck gene was downloaded from NCBI at the Nucleotide database (NM_033507). The strategies of Cloning that we use were based on [32]. Plasmid Constructs Human gck genomic sequences were amplified using the polymerase chain reaction (PCR) from human genomic DNA purchased from Promega (Wisconsin, USA).

Five sequences match specifically for the 5′ sequence of the liver gck gene. Sequences spanning bases; −1049 to +135, −753 to +135, −571 to +135, −345 to +135, and −161 to +135, which are relative to the liver-specific mRNA start site (Table 13.1). Each one was amplified with primers with HindIII and ScaI restriction enzyme digestion sites to the 5′ and 3′ primers, respectively, to have directional cloning. PCR products of Promoter fragments were purified by MinElute PCR Purification Kit (Qiagen) and cloned into a luciferase reporter plasmid pGL3Basic 3.1 (Promega, USA), which generated plasmids hGK-1049Luc, hGK-753Luc, hGK-571Luc, hGK-345Luc, hGK-161Luc. It was named hGK to differentiate it from the endogenous promoter.

A mutant’s site was generated using the shorter promoter vector to confirm the importance of the two E-box sites’ closest mRNA start site, hGK-161Luc. Site-directed mutagenesis protocol was used according to the protocol suggested for the kit GeneArt® Site-Directed Mutagenesis PLUS System and using the following specific primers designed with the mutation; hGK mutebox1/2_FW:5′-TCCCCG TGGCCTGTTGGCTTTACCTGGATGGCC TACCTC CCTTTC-3′ and hGK mutebox1/2_RV:5′-GAAAGGGAGGTAGGC CATCCAGGTA ACAGCCAACAGGGCACC GGGA-3′. All cloned promoter sequences were verified by sequencing. Enzyme restriction sequences are underlined.

Luciferase assays: Hep3B cells were seeded onto a 48-well plate at 80% of confluence for reporter assays. They were transfected the next day with indicated promoter-reporter plasmids, mutated reporter vectors, and over-expression plasmids using Lipofectamine 2000 (Invitrogen) method. The overexpression plasmids were BMAL1 (pBMPC3), CLOCK1 (pCKPC4), PER1 (pCMV Sport2 mPER1) and PER2 (pCMV Sport2 mPER2) purchased from Addgene (Massachusetts, USA). In order to normalize transfection efficiency, Renilla luciferase (pRL-TK; Promega) was used in transfections. Then, the Luciferase expression was measured after 48 h using the Promega Dual-Luciferase Reporter Assay System (Promega).

| Name | Location | Strand | Sequence | RE site |
|------|----------|--------|----------|---------|
| −1049f | −1049 | + | gcgcgaagCCACTTGGCCTCAGCTTCAGGC | SacI |
| −753f | −753 | + | gcgcgaagAGGGCCTTGGGAGGTGAT | SacI |
| −571f | −571 | + | gcgcgaagAGAGCCTGGAAAGTCAGGTC | SacI |
| −345f | −345 | + | gcgcgaagACCTCAAGAGCAAGTCACAG | SacI |
| −161f | −161 | + | gcgcgaagATCCCTACCCCATGTTCACAG | SacI |
| Ex1 | 135 | − | gcgcgaagTTTGGGAGGCAGAGATGCTCC | HindIII |
Statistical analysis: Statistical values were evaluated by one-way analysis of variance (ANOVA), followed by Dunnett’s multiple comparisons tests. The values of results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with the statistics package Test GraphPad, (Prism 8, GraphPad Software, San Diego, California, USA). In all cases, the significance level was set at \( P < 0.05 \); it was considered statistically significant. All experiments were performed with at least three individual replicates.

13.3 Results

Glucokinase expression follows a circadian profile/rhythm in rat hepatocytes in vitro

The first approach was to assess whether primary cultures of hepatocytes can maintain their circadian clock autonomously in vitro. RT-qPCR analysis was performed along with the periodic expression of core clock genes \( bmal1 \) and \( clock \) to determine whether \( gck \) mRNA may follow a circadian expression in primary rat hepatocytes in vitro.

Figure 13.1 shows that \( bmal1 \) and \( clock \) mRNA expression (Fig. 13.1a, b) have a circadian profile, with an increase in the expression levels from 0 h (circadian time) and a peak of expression after 4 h, followed by a noticeable decrease that reaches baseline expression levels after 8 h.

Similarly, \( gck \) mRNA expression (Fig. 13.1a) exhibited an oscillatory circadian profile reaching a peak of expression after 4 and a lower expression between 8 and 20 h. After 20 h, there was a rapid rise in mRNA expression for \( gck \). These results demonstrate that primary hepatocytes cultures express \( gck \) in a circadian profile and maintain their circadian rhythms through the mRNA expression of \( bmal1 \) and \( clock \).

\( bmal1 \) induces the Glucokinase circadian rhythmic expression in hepatocytes

In order to determine whether clock genes regulate the endogenous \( gck \) gene, an in vitro culture of primary rat hepatocytes with a circadian loss of function was generated by inhibiting \( bmal1 \) expression. First, it was analyzed the efficiency of transfection and inhibition of shRNA for \( Bmal1-GFP \) in the H4 cell line. Then, \( bmal1 \) mRNA was knocked down using an shRNA coupled to GFP expression to control transfection efficiency. Fluorescence analysis of cells transfected with indicated shRNAs for GFP expression showed an equivalent transfection percentage (\( \sim 40\% \)) of both \( Bmal1 \) shRNAs used (shRNA1 and shRNA2) (Fig. 13.2a–j). The inhibition efficiency of each \( Bmal1 \) shRNA was quantified by RT-qPCR assay; the plot showed effective inhibition of \( bmal1 \) by both siRNAs at 24 h in about 54% for shRNA1 and 44% for shRNA2 (Fig. 13.2k).

These results show that the shRNAs for \( bmal1 \) inhibit efficiently the expression of this endogenous cyclophilin amplified in the same sample and under the same experimental conditions. The relative temporal expression of each mRNA of interest was normalized to cyclophilin. \( \Delta Ct \) values were normalized to the average \( \Delta Ct \) of time 0 (0 h post-transfection). Data are the Mean ± SD of three independent experiments.
gene in H4 cell line and that the inhibition is more effective after 24 h of transfection.

Next, to determine whether clock genes control the gck mRNA expression, the sh-Bmal1-GFP shRNA1 construct was used to evaluate the loss of circadian expression in hepatocytes in vitro. Also, bmal1 and per2 mRNA expression were analyzed since both are transcription factors of the central core of the circadian clock. bmal1 and per2 mRNA expression were determined by RT-qPCR after using a specific shRNA against it. As it was expected, bmal1 and per2 mRNA expression showed an evident disruption in the circadian profile expression during 24 h (Fig. 13.3a, b).

Similarly, the gck circadian expression observed in the control conditions was severely decreased during the 4 h of circadian peaks in hepatocytes in vitro (Fig. 13.3c). On the other hand, the inhibition of the expression of bmal1 leads to a loss of the oscillatory circadian profile of gck. These results show that the downregulation of mRNA of bmal1 influences the expression of the gck gene.

Analysis of E-box regions in human and rat glucokinase promoter

It has been shown that the upstream sequences of the starting site of the gck transcription up to 1 Kb contain regulatory sequences essential for gene expression [32]. However, there is no evidence for specific sequences for the gck promoter, such as E-box, for its regulation through the core clock.

The structure of the gck gene has been characterized in mammals, rats, mice, and humans
Different regions of the promoter site have been proposed as transcriptional regulators in the three studied mammalian species [21, 32]. So, to determine sites where core clock proteins can bind to activate gck transcription through E-box, 5′ flanking sequence (−1049 to +135 bases) of the gck liver promoter (lgck) from rat and human species were analyzed. The analysis of E-box sites in the human gck promoter region (NM_033507) revealed the existence of nine non-canonical E-box consensus sequences (CANNTG) within the proximal minimal promoter at −82 and −93 relative to the transcription starting site and seven E-box non-canonical consensus sequences were identified at more distal sequences at −219, −483, −594, −611, −632, −668 and −758 (Fig. 13.4). Comparing the sequence to the rat lgck gene promoter (AH002177.2), eight E-box sites distributed throughout the 1049 bp analyzed within the proximal minimal promoter at −59 and −68 relative to the transcription starting site, and seven more distal sequences at −208, −449, −576, −866, −911 and −1038 were found (Fig. 13.4). At least 3 of these sites (E-box 1, 4 y 9) were located in similar human and rat lgck promoter regions.

Fig. 13.3 Down regulation of mRNA of bmal1 leads to a loss of the oscillatory circadian profile of gck and per2. Temporal expression analysis of bmal1, per2, and gck in bmal1 knockdown primary hepatocytes. The cell culture was transfected with shRNA for bmal1 or scramble (Control) 24 h before the first sample acquisition. After that, the samples were obtained every 4 h for 24 h and analyzed by qRT-PCR assay of mRNA for bmal1, per2, and gck in the presence of bmal1 shRNA (red lines) or scramble shRNA (black lines) to obtain a temporal expression profile of each gene. The relative mRNA expression of each gene of interest was normalized to Cyclophilin. ΔCt values were normalized to the average ΔCt of time 0 (0 h post-transfection). Data are the Mean ± SD of three independent experiments.

BMAL1 and CLOCK regulate the transcriptional activity of human glucokinase promoter

To evaluate the contribution of E-box sites in the 5′ flanking sequence (−1049 to +135 bases) of human liver-specific gck promoter, a series of reporter constructs upstream of the luciferase gene with different lengths of the 5′ flanking sequence was generated. The promoter activity of the constructs was tested in the human hepatoma cell lines Hep3B. All promoter fragments constructs showed a discreet basal activity when transfected in Hep3B (Fig. 13.5a, black bars). The co-transfection of reporter constructs with CLOCK and BMAL1 significantly enhanced reporter gene expression in all the promoter constructs (Fig. 13.5a, dark grey bars). These results confirmed that the gck promoter contains significant regulatory regions for gene transcription. Human hGK-161Luc exhibited a higher transcriptional response to CLOCK and BMAL1. This fragment includes 2 E-box sites of interest; one (E-box1) is highly conserved in rats and humans (Fig. 13.4). In turn, per1 and per2 ectopic expression did not modify the basal levels of all promoter fragment constructs (Fig. 13.5a, gray light bars) while significantly
reducing the capacity of clock and bmal1 to enhance the transcriptional activity of human hGK-161Luc hGK-345Luc and hGK-571Luc constructs (Fig. 13.5a white bars). Next, the contribution of proximal E-box localized at −82 and −93 to the lgck transcriptional activity was determined. The two e-box sites (EB-1 and EB-2) in construct hGK-161Luc were mutated (hGK-161mut-Luc) and subjected to transcriptional regulation by clock genes (Fig. 13.5b). Transient transfection of Hep3B cells with the hGK-161mut-Luc revealed an increase of 104-fold in promoter activity compared to the wild-type promoter construct. At the same time, hGK-161mut-Luc activity was decreased by about 1.5-fold by CLOCK/BMAL1 and about 1.7-fold by PER1/PER2 expression. However, the ectopic co-expression CLOCK/BMAL1 and PER1/PER2 hGK-161mut-Luc did not modify PER2 hGK-161mut-Luc promoter activity.

In summary, it was cloned the human gck promoter to characterize its dependence on CLOCK/BMAL1 and PER1/PER2, in particular via E-boxes. The hGK promoters were active in Hep3B. Moreover, in the hepatoma cell lines, the hGK promoters activity was increased by cotransfected BMAL1/CLOCK but were not affected by co-transfected PER1/PER2. Thus, BMAL1/CLOCK modulate human gck promoter activity, most likely via E-boxes.

13.4 Discussion

The circadian clock of the liver has been reported to regulate metabolism and energy homeostasis. The availability of glucose in the body is a measure of its energy state and therefore determines the ability of cells to work correctly. Deregulation of this process causes important diseases such as obesity and diabetes. Peripheral organs that are involved in the maintenance of glucose homeostasis include the liver. However, few studies have focused on elucidating the basis of this process and how the circadian clock is involved.

The regulation of glucose homeostasis is one of the liver’s leading roles in mammals’ metabolism. Namely, the glucose transporter GLUT2, a BMAL1-regulated transporter [7], enters glucose into hepatocytes and rapidly converts it into
glucose-6-phosphate by GCK, which also shows a rhythmic expression. Liver GCK expression regulates glycolysis and glycogen synthesis pathways. It shows a rhythmic pattern reaching maximum alongside the transition from the rest phase to the active phase of the feeding–fasting cycles [34].

Although there is consensus on the importance of the enzyme GCK in metabolic processes and diseases, the regulation of gck expression and transcriptional mechanisms is unclear [22]. Interestingly, the circadian expression profiles of genes involved in glucose regulation mechanisms in the liver coincide with their metabolic functions. For example, the glucose transporter GLUT2, presents its maximum expression during the fasting phase of the cycle. In contrast, GCK shows its maximum circadian expression during the feeding phase [7].

This research allows an understanding of the specific mechanisms associated with the regulation of circadian expression of gck in the liver cells. In isolated rat hepatocytes, the results indicated that gck exhibits an oscillatory circadian pattern similar to those observed for bmal1 and clock (Fig. 13.1).

Glucose homeostasis is one of the leading liver functions regulated by circadian control. In this line, it has been demonstrated that glucose transporter 2 (GLUT2) is regulated by clock BMAL1 through E-box elements. The figure illustrates the effect of E-box mutations on gck promoter activity in Hep3B cells. E-box sites at −211/−222 were mutated in combination using site-directed mutagenesis. Hep3B cells were transfected with hGK-mut-luc or hGK-luc constructs in different conditions; co-transfected with clock/bmal1, co-transfected with per1/per2, and co-transfected with clock/bmal1/per1/per2. Firefly luciferase expression was measured at 48 h post-transfection and normalized to renilla luciferase to correct for variability in transfection efficiency. Results are expressed as the mean of four independent experiments, each one performed in triplicate.
hepatocytes directly impacts the expression of clock genes and the glucose metabolism-related glycolytic metabolic pathways, glycogen metabolism, and gck gene. Consequently, the liver function in maintaining glucose blood levels through glycolysis is critically implicated in all those functions.

Our results showed that gck is a dependent circadian gene, and a functional cis-acting element of the E-box type was identified in the liver promoter of the gck gene. Similar to GLUT2, gck exhibits an oscillatory circadian pattern similar to bmal1 and clock.

It has been suggested that core clock genes and gck mRNA can maintain an autonomous circadian rhythm in vitro, independent of the central clock and other local controls such as hormonal and neural signals [7]. In vivo evidence demonstrated that gck expression has a circadian regulation since its rhythmic expression is lost in bmal1 knockout mice [7]. However, this research could not demonstrate whether this circadian control is autonomous or dependent on the central clock. In our study, we found a circadian expression profile in a culture of hepatocytes isolated from the central control, which suggests that it behaves as an autonomous clock in culture.

Our results also confirms the critical role of bmal1 on the gck rhythmic expression since its knockdown inhibits gck and per2 circadian profile expression. These results suggest that a loss of function in the central clock through bmal1 in hepatocytes directly impacts the expression of clock genes and the glucose metabolism-related gck gene. Consequently, the liver function in maintaining glucose blood levels through glycolysis metabolic pathways, glycogen metabolism, and glycogenesis [36] could be further affected since GCK is critically implicated in all those functions.

Consequently, knowing the relevance of the gck gene regulation for its function in hepatocytes and due to the lack of a proposed mechanism to date, the next question was how clock genes regulate gck expression at the promoter level. In this sense, the cyclic activation and repression of E-box-driven transcription seem to be one of the primary mechanisms for circadian gene expression [37]. Accordingly, in silico analysis showed the presence of several E-boxes within rat and human gck promoters (Fig. 13.4). The results demonstrated that the activity of the 5 fragments of the human gck gene studied (1049, 753, 571, 345, and 161 bp) increases in the presence of CLOCK and BMAL1 in vitro (Fig. 13.5). This data suggest that the sequence established in this region of the gck gene contributes to the positive regulation of the gene in the presence of CLOCK and BMAL1. Contrary to expected, the highest activation was found in the smallest fragment, hGK161. At the same time, the site-directed mutation of 2 E-box sequences in the 161 gck fragment provided consistent evidence of the existence of that regulatory region at the gck promoter. The latter is of utmost importance given that if these 2 E-box sequences are eliminated, the activity of the reporter gene increases significantly regarding the control and the activation with BMAL1 and CLOCK. This suggests that two E-boxes sites proximal to the starting transcription site may have a repressive function on the gck promoter. In addition, the activation-inhibition degree can also implicate the clock proteins’ affinity with the site or binding sequence [38].

This study revealed the complexity of the gck gene regulatory mechanism associated with clock proteins. It opened the question of which specific sequences within this 1049 bp contribute to this inhibition and what other endogenous proteins can be involved. In this respect, there is evidence of several transcription factors and regulatory elements (USF2, HNF4, HIF1A, among others) associated with this first fragment of gck that can intervene in gene regulation, but that is not the focus of this study (or are not evaluated in this research) and further studies are necessary to elucidate it (review by [21]).

On the other hand, positive and negative regulatory elements have been demonstrated within the gck human gene sequence. More specifically, several inhibitory sites above the 1049 bp were detected in cells L-02 and HepG2 [32] that contribute to the “down or up-regulation” of the gene basal expression in the
presence or absence of insulin or serum. In this study, it is worth mentioning that the authors compared two cell lines that presented different behaviors in all the studied conditions. This indicates that the expression environment (endogenous factors) is relevant for the gene response activation, as we mentioned before.

In summary, different fragments of the gck promoter that are proximal to the transcription starting site and contain E-box sequences are active when incorporated into the Hep3B cell line. The basal activity was increased by clock/bmal1 co-transfection but inhibited by per1/per2 co-transfection. We can conclude that clock proteins modulate the human gck promoter transcriptional activity. In line with previously reported results, our data suggest that the circadian clock plays a crucial role in regulating the temporal expression of gck.

To complete the definition of this complex circadian regulation of gck promoter, it could be interesting to involve in vitro studies on other peripheral tissues implied in glucose homeostasis, such as the pancreas. Also, include in vivo models where those tissues are connected by the central clock.

**Statements and Declarations**

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**Ethical Approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Approval was granted by the Ethics and Animal Care and Use Committee of the National Research and Development Agency (ANID, No. 1221508) and the Universidad de Concepción, Chile.

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Part III

Molecular Pathology of Cancer: Determinants and Potential Therapies
Correlation Between Endoglin and Malignant Phenotype in Human Melanoma Cells: Analysis of hsa-mir-214 and hsa-mir-370 in Cells and Their Extracellular Vesicles

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Abstract

Endoglin (CD105) is an auxiliary receptor of transforming growth factor (TGF)-β family members that is expressed in human melanomas. It is heterogeneously expressed by primary and metastatic melanoma cells, and endoglin targeting as a therapeutic strategy for melanoma tumors is currently been explored. However, its involvement in tumor development and malignancy is not fully understood. Here, we find that endoglin expression correlates with malignancy of primary melanomas and cultured melanoma cell lines. Next, we have analyzed the effect of ectopic endoglin expression on two miRNAs (hsa-mir-214 and hsa-mir-370), both involved in melanoma tumor progression and endoglin regulation. We show that compared with control cells, overexpression of endoglin in the WM-164 melanoma cell line induces; (i) a significant increase of hsa-mir-214 levels in small extracellular vesicles (EVs) as well as an increased trend in cells; and (ii) significantly lower levels of hsa-mir-370 in the EVs fractions, whereas no significant differences were found in cells. As hsa-mir-214 and hsa-mir-370 are not just involved in melanoma tumor progression, but they can also target endoglin-expressing endothelial cells in the tumor vasculature, these results suggest a complex and differential regulatory mechanism involving the intracellular and extracellular signaling of
hsa-mir-214 and hsa-mir-370 in melanoma development and progression.

**Keywords**
Cancer • Melanoma • miRNAs • Extracellular vesicles • Exosomes • Endoglin • TGF-β • BMP

**Abbreviations**

| Abbreviation | Full Form |
|--------------|-----------|
| AKT          | Serine/threonine-specific protein kinase B (PKB) |
| ALIX         | ALG-2-interacting protein X |
| BMP          | Bone morphogenetic protein |
| BSA          | Bovine serum albumin |
| CAFs         | Cancer-associated fibroblasts |
| circRNA      | Circular RNA |
| CMV          | Citomegalovirus |
| CYLD         | Cylindromatosis |
| DMEM         | Dulbecco’s modified Eagle medium |
| EMT          | Epithelial-mesenchymal transition |
| EVs          | Small extracellular vesicles |
| FBS          | Fetal bovine serum |
| HA           | Hemagglutinin |
| HEPES        | 4-[2-Hydroxyethyl]-1-piperazineethanesulfonic acid |
| HHT          | Hereditary hemorrhagic telangiectasia |
| hPSCs        | Human pancreatic stellate cells |
| HRP          | Horseradish peroxidase |
| hsa-mir      | Homo sapiens microRNA |
| MAFs         | Melanoma-associated fibroblasts |
| MEK          | Mitogen-activated ERK kinase |
| mir          | MicroRNA |
| miRNA        | MicroRNA |
| NP-40        | Nonidet P-40 |
| PAGE         | Poly-acrylamide gel electrophoresis |
| PBS          | Phosphate-buffered saline |
| PMEL         | Premelanosome protein |
| qRT-PCR      | Real-time quantitative reverse transcription PCR |
| SDS          | Sodium dodecyl sulfate |
| TAMs         | Tumor-associated macrophages |
| TBS          | Tris-buffered saline |
| TGF-β        | Transforming growth factor beta |
| TYR2         | Tyrosinase 2 |

### 14.1 Introduction

The mechanism involved in tumor development and dissemination of cancer cells is still poorly understood and numerous proteins, miRNAs and signaling pathways have been reported to regulate this process [1, 2]. Among these, endoglin, an auxiliary receptor of the transforming growth factor β (TGF-β) family, has emerged as a promising therapeutic target [3, 4]. Endoglin (Eng; CD105) is a 180-kDa disulfide-linked homodimeric transmembrane glycoprotein [5, 6] highly expressed by proliferating endothelial cells in tumor associated neoangiogenesis [7], as well as in a large number of cancers with poor prognosis [8–13]. The role of endoglin in tumor progression and metastasis has been studied in several cancer cell types using in vitro and in vivo models [14–21]. In this regard, an active role of endothelial endoglin in extracellular extravasation of healthy and metastatic tumor cells has been postulated [22, 23]. Furthermore, endoglin-targeted therapy for malignant melanoma is currently been investigated with promising results [24–26]. While endoglin is heterogeneously expressed by primary and metastatic melanoma cells, its involvement in the malignant and metastasis processes is not fully understood [8, 27–30]. Given the high mortality rate of this type of skin cancer and the unresponsiveness of some patients to current immunological treatments, a better knowledge of the mechanisms and active players involved in melanoma growth and development, including endoglin, is a subject of scientific and clinical interest [31, 32].
While endoglin is a type 1 transmembrane glycoprotein with cytoplasmic, transmembrane and extracellular regions, almost 90% of the protein is encompassed within its extracellular region [5]. For this reason, the extracellular region of endoglin has focused many structural and functional studies [6, 33]. Structurally, the extracellular region of endoglin contains two distinct domains: (i) a conserved Zona Pellucida (ZP) juxtamembrane domain at the C-terminus consisting of ~260 amino acids (Lys362-Asp561) with eight conserved cysteine residues and divided in two well-defined subdomains (ZP-C and ZP-N); and (ii) a domain at the N-terminus named orphan (orphan domain; OD) due to its lack of significant homology with other protein families [34, 35]. The orphan domain is involved in recognition of TGF-β family ligands [35, 36], whereas the ZP domain is involved in the interaction with members of the integrin family via its arginine-glycine-aspartic acid (RGD) motif located within the ZP-N subdomain [37]. The cellular and pathophysiological function of endoglin has been widely studied in endothelial cells, which are the target in hereditary hemorrhagic telangiectasia type 1 (HHT1), a vascular disease caused by heterozygous mutations in the endoglin gene. HHT1 is associated with telangiectases in skin and mucosa, as well as with arteriovenous malformations in lung, liver, and brain [38, 39]. As an auxiliary receptor of the TGF-β system, endoglin can bind with high affinity to bone morphogenetic protein (BMP)-9 and BMP-10 ligands [36] and interact with the type I and II serine/threonine kinase TGF-β receptors, including ALK1 and ALK5 (type I receptors) and the type II TβRII [40, 41] to modulate cellular responses to different TGF-β family members. Several lines of experimental evidence suggest that binding of BMP9 to endoglin potentiates ALK1 signaling, including the fact that mutations in the gene coding for ALK1 (ACVRL1) are responsible for a second form of HHT (HHT2), whereas heterozygous and homozygous mutations in GDF2, the gene encoding BMP-9, lead to an HHT-like variant [38, 42]. Signaling triggered by BMP-9 through the endoglin/ALK1 route mediates, via the Smad1/5/8 pathway, the expression of a wide range of genes, including the gene for the helix-loop-helix transcription factor inhibitor of differentiation 1 (ID1), a negative transcriptional regulator which is involved in the development of malignant melanoma [43–45]. Beyond the TGF-β/BMP-related functions, endoglin is also involved in integrin-mediated cell adhesion via its RGD motif in its extracellular ZP-N subdomain. Thus, endoglin has shown functional binding activity to integrins, such as α5β1 or αIIbβ3 from leukocytes, smooth muscle cells and platelets [22, 37, 46]. Of note, integrins, the major family of cell adhesion receptors in humans, play a key role in tumor growth and metastasis and several studies have investigated the contribution of integrins to the phenotypic aggressiveness of melanoma [47, 48]. In this line, differential expression of integrins in primary cutaneous melanoma has been used to distinguish indolent from aggressive, prometastatic melanoma. Also, some integrins preferentially direct circulating melanoma cells to specific organs, promoting the development of metastases. For example, melanoma cells expressing β1 or β3 integrins, both endoglin interactors, tend to metastasize to the lungs or generate lymph node metastases, respectively. In addition to their relevant role in mediating invasion and metastasis, integrins are not only promising biomarkers, but also attractive therapeutic targets in melanoma [47, 48]. Given the role of integrins in tumor angiogenesis, tumor cell migration and proliferation, and organ-specific metastasis in malignant melanoma, it can be postulated that endoglin, as integrin counter-receptor, will have a relevant impact in melanoma development.

In addition to the membrane-bound form of endoglin, a circulating form of endoglin packed into small extracellular vesicles (EVs) has been described in several pathological conditions, such as preeclampsia, liver disease or thromboembolic pulmonary hypertension [49–52]. Heterogeneous EVs, including exosomes, can be secreted by all cell types carrying various bioactive cargos such as proteins, RNAs, lipids or metabolites [53].
They are emerging as key regulators of intercellular communication in health and disease with potential relevance as biomarkers and therapeutic strategies in different pathological conditions [54, 55]. EVs can transfer their bioactive cargo from donor to recipient cells and influence the biological function of the target cell. In this regard, a functional role for circulating endoglin in EVs has been postulated in several studies, including a protective mechanism supporting endothelial cell survival and angiogenesis [49]. In addition, endoglin⁺ EVs have been proposed as a biomarker for preeclampsia and metastatic breast cancer [10, 50]. Among the different bioactive cargos of EVs are microRNAs (miRNAs, miRs), which are small endogenous non-coding RNAs that regulate gene expression. During the last decade, compelling evidences support the involvement of cellular and EVs miRNAs in cancer. Among others, miRNAs may act as either tumor suppressors or oncogenes, activating invasion and metastasis, or inducing angiogenesis; as therapeutic targets; and as potential biomarkers for cancer diagnosis, and prognosis [56–59]. Aberrant expression of miRNAs occurs in several human cancers, including melanoma. Thus, dysregulation of miRNAs has been linked to suppression, progression, differentiation, development, and prognosis of melanoma [60–62]. Some miRNAs are specific for one or more skin cancer type, such as hsa-mir-21 and hsa-mir-221, which are observed in cutaneous melanoma and squamous carcinoma; while hsa-mir-155 has been detected in melanoma and cutaneous lymphoma. In this work, we have focused our studies on the pleiotropic hsa-mir-214 and hsa-mir-370, as they are predicted and have been shown to target endoglin [63, 64]. Both, hsa-mir-214 and hsa-mir-370 are dysregulated in several other tumors, besides skin cancers, displaying contrasting behavior. Regardless of whether hsa-mir-214 levels are upregulated or downregulated in skin cancer and melanoma, its dysregulation always correlates with metastasis or poor progression [65, 66]. In the case of hsa-mir-370, controversial findings have also been reported since its upregulation correlates with progression and poor prognosis in breast and prostate cancer [67, 68], as well as promotion of cell apoptosis and inhibition of proliferation in human gastric cancer [69]. By contrast, (i) downregulation of hsa-mir-370 in esophageal squamous-cell carcinoma is associated with cell proliferation and cancer progression [70], and (ii) hsa-mir-370 acts as a tumor suppressor in hepatocellular carcinoma [71]. Interestingly, enforced expression of hsa-mir-370 in melanoma cell lines promotes proliferation, inhibits apoptosis and enhances invasion [72]. Overall, these contradictory results suggest that the function of these miRNAs is highly dependent on the cancer cell context, likely due to their differential cell source, cell target, expression level and/or specific mRNA targeting in each case.

Here we have delved into role of endoglin in human melanoma. We find a correlation between expression levels of endoglin with malignancy in primary melanomas and cultured melanoma cell lines. In addition, overexpression of endoglin in a melanoma cell line leads to dysregulated levels of hsa-mir-214 and hsa-mir-370, mRNAs involved in melanoma tumor progression and endoglin regulation. These results suggest that endoglin is actively involved in development and dissemination of malignant melanoma, and identify endoglin as a potential therapeutic target to block tumor progression.

14.2 Methods

Immunohistochemistry of melanoma tissues: A total of 73 human specimens (3 benign nevi, 73 malignant melanomas) were analyzed with the corresponding informed consent and ethical protocols approved by the Clinical Investigation Ethical Committee. Immunohistochemistry was performed on 4-μm-thick sections of formalin-fixed, paraffin-embedded tissue samples using an anti-endoglin monoclonal antibody (SN6h, Dako). The staining results were independently analyzed by two expert pathologists who were blinded to the staging and clinical features of the subjects.
Cell culture: WM-164, SK-Mel-28, SK-Mel-103, and SK-Mel-147 cell lines were kindly provided by Dr. Maria S. Soengas (Spanish National Cancer Research Centre (CNIO), Madrid, Spain). Cells were cultured in DMEM (Lonza BE12-604F) supplemented with 10% heat-inactivated filtered fetal bovine serum (FBS) (Gibco) and 20 µg/mL gentamycin (Lonza 17-519Z). This melanoma cell line was routinely tested for mycoplasma contamination.

Lentiviral production and generation of human ENG stably overexpressing WM-164 cells: Lentiviral plasmids expressing human endoglin containing a hemagglutinin (HA) tag (pLV-CMV-IRES-Puro/hEng) and the corresponding empty vector (pLV-CMV-IRES-Puro/Ø) were kindly provided by Professor Peter ten Dijke (LUMC, Leiden, The Netherlands). These vectors were used in conjunction with the packaging plasmids p8.91 and pSVCG. HEK 293T cells were seeded in a 10-cm plate and transfected with 5 µg p8.91, 2.5 µg pSVCG and 5 µg pLV-CMV-IRES-Puro/Ø or pLV-CMV-IRES-Puro/hENG, using Lipofectamine® 2000 (Thermo Fisher Scientific), according to the manufacturer’s instructions. After 10–12 h, medium was changed by fresh culture medium (DMEM) and cells were incubated for additional 48 h. Culture supernatants containing lentiviral particles were harvested, clarified by centrifugation at 1500 rpm for 5 min, and filtered through a 0.45 µm filter. Lentiviral particles at 1:3 dilution were used to infect WM-164 cells in suspension in the presence of 4 µg/mL polybrene (Sigma). After incubation for 24 h, medium was replaced by fresh culture medium. Twenty four hours later, infected cells were selected in the presence of 0.4 µg/mL puromycin (Sigma), and the resulting endoglin-overexpressing WM-164 cells (WM-164 ENG) were validated by immunoblot and flow cytometry analyses.

Immunoblot assays: Cells were washed twice with PBS and lysed in cold lysis solution containing 50 mM HEPES pH 7.5, 0.4 M KCl, 10% glycerol, 1% NP-40 and protease inhibitors (PhosSTOP™, Sigma Aldrich). Lysates were sonicated for 1 min and centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatant fractions were used for Western blot analyses. Protein extracts or purified EVs were quantified for protein content using the bicinchoninic acid assay (Pierce™ BCA Protein Assay kit, Thermo Scientific). Equal amounts of extracted protein or purified EVs from each sample were resuspended in Laemmli buffer and, subsequently, incubated at 95 °C for 10 min. Samples were separated by SDS-PAGE and then transferred onto a PVDF membrane (Invitrogen). Protein-bound membranes were blocked with 0.1% Tween-20 (Sigma-Aldrich) in Tris-buffered saline (TBS) containing 5% BSA or 2.5–3% milk (TBS-T), and phosphatase inhibitor cocktail (0.2 mM sodium orthovanadate, 5 mM sodium beta-glycerophosphate and 10 mM sodium fluoride) for 2 h at room temperature. Membranes were then incubated overnight at 4 °C with the following primary antibodies specific for: human endoglin (1:1000 in TBS-T/BSA, Abcam #169545); ALIX (1:1000 in TBS-T/milk, Cell Signal #2171); MEK 1/2 (1:1000 in milk; Cell Signaling #8727S); AKT (1:1000 in milk, Cell Signaling #9272); β-actin (1 µg/mL in TBS-T/BSA, Sigma #A1978); and GAPDH (1:500 in TBS-T/BSA, Abcam #9484). Then, membranes were washed with TBT-T and incubated for 1 h at room temperature with the corresponding secondary HRP-linked antibodies. After rinsing with TBS-T, protein bands were revealed using SuperSignal™ West Pico PLUS Chemiluminescent substrate (Thermo Scientific) to enhance HRP luminescence, followed by analysis using the Molecular Imager® Gel Doc™ XR+ System with Image Lab™ software (Bio-Rad).

Immunofluorescence flow cytometry: Cell surface expression of endoglin in WM164 cells was analyzed by flow cytometry. After collecting and washing transfected cells in PBS by soft centrifugation at 1000 rpm 8 °C for 5 min, non-specific binding was blocked for 20 min at 4 °C with sterile-filtered 1% BSA in PBS (PBS-BSA). Cells were then incubated for 1 h at 4 °C with a mouse monoclonal antibody against human endoglin (P4A4, anti-CD105, 1/100; Developmental Studies Hybridoma Bank-DSHB-
University of Iowa, USA) or against the hemagglutinin (HA) tag (1/100; MilliporeSigma). As a negative control, cells were stained with isotype control antibodies (Immunostep, Salamanca, Spain) at the same concentration as the corresponding primary antibody. Following incubation with primary antibodies, cells were washed with PBS, and incubated with Alexa-Fluor-488-conjugated anti-mouse antibody (1/200, Molecular Probes) for an additional period of 45 min. Samples were then washed, resuspended in cold PBS, and analyzed with a FC500 Beckman Coulter flow cytometer using the FlowLogic software. Endoglin protein levels were measured using the fluorescence intensity mean and expressed as fold induction relative to empty-transfected cells.

**EVs isolation by sequential ultracentrifugation, characterization and analyses:** Cells were cultured in media supplemented with 10% EVs-depleted FBS. Serum was depleted of bovine EVs by ultracentrifugation at 100,000 g for 70 min at 10 °C and then filtered. Supernatant fractions collected from 48 to 72 h exponentially growing cell cultures were pelleted by centrifugation at 500 g for 10 min at 4 °C to remove any cell contamination. In addition, possible apoptotic bodies and large cell debris were removed from supernatants by centrifugation at 12,000 g for 20 min at 10 °C. EVs, including exosomes were then collected by spinning at 100,000 g for 70 min at 10 °C. The pellet with EVs was then washed in 20 mL of PBS and collected again by ultracentrifugation at 100,000 g for 70 min at 10 °C (Beckman, L100 X-P). The final pellet of EVs was resuspended in PBS. EVs size and particle number were analyzed using Nanosight (Nanoparticle Tracking Analysis-NTA) and its protein content was measured by BCA.

**RNA isolation, cDNA synthesis and quantitative RT-PCR:** microRNA (miRNA) and total RNA were isolated from cells using the miRNeasy Micro kit (Qiagen), according to the manufacturer’s instructions. To quantify specific microRNAs, first they were reversed transcribed using Taqman™ MicroRNA Reverse Transcription kit and then, PCR was performed using Taqman Universal PCR Master mix (Applied Biosystems) and specific and pre-designed Taqman® MicroRNA assays (hsa-miR-214: ID 002306 and hsa-miR-370: ID 002275). For quantification of gene expression, RT-PCR was performed with SuperScript™ II (Invitrogen) and FastStart Essential DNA Green Master (Roche) using the primers shown in Table 14.1. qRT-PCR was performed on Light Cycler 96 (Roche), according to the following PCR settings: initial denaturation for 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C for miRNA assays and 30 s at 60 °C in the case of gene expression assays. Both miRNA and total RNA quantifications were performed in triplicates. Gene and miRNA expressions were analyzed using the delta-deltaCT method for relative quantification and all samples were normalized to the corresponding housekeeping gene, hsa-miR-16 ID 000391 and human mRNA β-actin.

### 14.3 Results

**Endoglin expression in primary melanomas and cultured cells**

Endoglin expression was assessed in a cohort of primary melanomas and dermal nevi by immunohistochemistry. As expected, staining of endoglin was observed in endothelial cells from primary melanomas and dermal nevi. While endoglin staining was not detected in dermal nevi, 41.4 and 18.6% of primary melanomas showed low or high endoglin expression, respectively, in tumor cells (Fig. 14.1). These data suggest that, compared to normal nevi, melanoma tumors show markedly increased levels of endoglin. This prompted us to study whether endoglin expression correlates with melanoma malignancy or metastatic potential. Endoglin expression levels were also analyzed by immunoblotting in a panel of different melanoma cell lines (Fig. 14.2). While the non-metastatic or low metastatic melanoma cell lines (WM-164 and SK-Mel-28, respectively) showed low levels of endoglin, the more metastatic cell lines (SK-Mel-147 and SK-Mel-103)
had higher levels of endoglin expression (Fig. 14.2). These results suggest that endoglin expression correlates with malignancy in primary melanomas as well as in cultured melanoma cell lines.

**Table 14.1**  Sequences of primers used for qRT-PCR

| Human gene | Sequence (5’-3’) |
|------------|-----------------|
| ENG (forward) | CTGCTGCTGAGCTGAATGAC |
| ENG (reverse) | AGTCCACCTTCACCCTGAC |
| PMEL (forward) | CTCATTCCAGTCGCACTTC |
| PMEL (reverse) | CAGATAGCCACTGGGTAC |
| TYR2 (forward) | TACGGCGTAATCCTGGAAA |
| TYR2 (reverse) | ATTTGCCATGCTGCTTGAA |
| MLANA (forward) | GCTCATCGGGCTGGTGTATT |
| MLANA (reverse) | ATAAGCAGGTGGAGCAATG |
| MITF (forward) | AACTCATGCGGTGAGCAAT |
| MITF (reverse) | TACCTGCAGGTGGAGCAAT |
| VEZF-1 (forward) | AGAGGAAAGCCGGCTGACT |
| VEZF-1 (reverse) | ACTCAGGAGCTTCACAGA |
| ACTB (forward) | GGACTTCGAGCAAGAGAT |
| ACTB (reverse) | AGCACTGTGTGGCGTAGA |

**Fig. 14.1**  Endoglin expression in primary melanomas. The presence of endoglin in human dermal nevi (n = 3) and primary melanoma (n = 70) tissues was analyzed by immunohistochemistry and endoglin staining was quantified and classified as negative, low intensity or high intensity by the pathologist.

| Primary melanomas | N (%) |
|-------------------|-------|
| Negative          | 28 (40.0) |
| Low intensity     | 29 (41.4) |
| High intensity    | 13 (18.6) |
| Total             | 70     |

| Dermal nevi | N (%) |
|-------------|-------|
| Negative    | 3 (100) |
| Low intensity | 0   |
| High intensity | 0   |
| Total       | 3     |

**Characterization of ectopically overexpressed endoglin in the WM-164 cell line**

To investigate the impact of endoglin in the malignant phenotype of melanoma cells, the low metastatic melanoma cell line WM-164 was...
transduced with a lentivirus encoding HA-tagged human endoglin. Following cell infection and puromycin selection, we verified the ectopic expression of endoglin by immunoblot analysis in cellular extracts and EVs fractions (Fig. 14.3a). As expected, endoglin-transduced cells and derived EVs showed a clear signal of ectopic endoglin relative to mock-transduced cells. To confirm the correct isolation of the EVs, the expression of ALIX, a broad biomarker of EVs, was tested. ALIX was not detected in cellular extracts, whereas a weak band was observed in EVs from mock- and endoglin-transduced cells (Fig. 14.3a), confirming the proper quality of purified EVs. To assess whether endoglin overexpression could be affecting other relevant signaling pathways, we analyzed total protein levels of MEK and AKT by immunoblotting. No significant differences were found in MEK and AKT protein levels between mock- and endoglin-transduced cells (Fig. 14.3b). The expression of ectopic endoglin in transduced WM-164 cells was also analyzed by flow cytometry using anti-HA or anti-endoglin (P4A4) monoclonal antibodies (Fig. 14.4). The strong expression of cell surface endoglin was demonstrated in endoglin-transduced WM-164 cells compared to the weak labelling of mock-transduced cells, as evidenced by the histograms obtained with anti-HA (Fig. 14.4a, left panel) and P4A4 anti-endoglin (Fig. 14.4a, right panel and Fig. 14.4b) monoclonal antibodies. Taken together, immunoblot (Fig. 14.3) and flow cytometry (Fig. 14.4) analyses demonstrate that

**Fig. 14.2** Endoglin expression in primary melanomas and melanoma cell lines. Western blot analysis (upper panel) of endoglin expression in four melanoma cell lines with different metastatic phenotype. Quantification of endoglin staining relative to β-actin is shown in the lower panel.
lentiviral transduction of WM-164 cells efficiently yields endoglin overexpression at their cell surface. The results from Figs. 14.1 and 14.2 suggesting that endoglin expression correlates with malignancy of melanomas prompted us to analyze the malignant phenotype of the endoglin-expressing WM-164 cells by measuring the levels of PMEL and TYR2, two well-known melanoma markers. PMEL (Premelanosome protein) is expressed by melanocytes and melanoma cells, and is widely used as a melanoma marker in serum samples. Compared with normal melanocytes, PMEL is over-expressed at all stages of melanoma progression [73, 74]. Tyrosinase TYR2 is involved in melanogenesis and mediates anti-apoptotic effects in human melanoma cells [75]. Analysis by qRT-PCR of endoglin-transduced WM-164 cells and their

**Fig. 14.3 Western blot analysis of endoglin-expressing WM-164 cells.** a Analysis of cellular lysates and EVs from mock-transduced (ø) or endoglin-transduced (hENG⁺) WM-164 cells using antibodies to endoglin (anti-HA), the EVs marker ALIX or GAPDH, as a loading control. b Analysis of cellular lysates from mock-transduced (ø) or endoglin-transduced (hENG⁺) WM-164 cells using antibodies to endoglin (anti-HA), total MEK, total AKT or β-actin, as a loading control. Representative Western blots are shown.

**Fig. 14.4 Flow cytometry analysis of endoglin-expressing WM-164 cells.** a Cell surface expression of endoglin was analyzed in mock-transduced (ø) or endoglin-transduced (hENG⁺) WM-164 cells using anti-HA or P4A4 antibodies that recognize the recombinant endoglin. Representative flow cytometry histograms are shown. b Endoglin protein levels were measured in cells stained with P4A4 anti-endoglin antibody (n = 4 per condition) as in panel (a). **p < 0.01; by two-tailed student’s t-test.
derived EVs showed that *PMEL* and *TYR2* mRNA levels were significantly higher than those of mock-transduced WM-164 controls (Fig. 14.5). These results further support the involvement of endoglin in melanoma progression.

**Effect of endoglin expression on additional markers of melanoma cancer cells**

To further assess whether endoglin upregulation exerted a functional effect during melanoma development and progression, three additional markers were analyzed: (i) MLANA (also known as MART-1, Melanoma antigen recognized by T-cells 1); (ii) MITF (Microphthalmia-associated transcription factor); and (iii) VEZF-1 (Vascular endothelial zinc finger 1). Of note, MLANA and MITF are relevant proteins involved in melanocyte and melanoma biology. MLANA is a cytoplasmic protein expressed by normal melanocytes and benign nevi and it is used in the clinic to detect and confirm melanocytic tumors [76, 77]. In addition, MITF has been described as the main transcription factor regulating key processes in melanoma cell development, growth, survival, proliferation, differentiation and invasion [78, 79]. Also, VEZF-1 is a Krüppel-like zinc finger protein that contributes to cancer pathogenesis [80, 81]. qRT-PCR analysis showed that MLANA, MITF and VEZF-1 mRNA levels were significantly increased in endoglin-expressing WM-164 cells, but not in EVs, both compared to mock-transduced cells (Fig. 14.6). These findings further support the active role of endoglin in melanoma development and progression.

![Fig. 14.5 Analysis of the melanoma marker genes PMEL and TYR2 in endoglin-expressing WM-164 cells.](image-url)

Cells and EVs showing the qRT-PCR results for *PMEL* and *TYR2* from cells (upper panels) or their derived EVs (lower panels) are shown (n = 3 per condition). *p < 0.05; **p < 0.01; ***p < 0.001; by two-tailed student’s t-test.
Effect of ectopic endoglin expression in the levels of hsa-miR-214 and hsa-miR-370

Emerging evidence support the involvement of cellular and EVs miRNAs in cancer progression, diagnosis, and prognosis [56–59], including melanoma [60–62]. Therefore, we investigated the effect of endoglin in the levels of miR-214 and miR-370 as they (i) have been found dysregulated in several cancer types, including skin cancers, (ii) are predicted to target endoglin [64], and (iii) differential expression of circulating miR-370 has been reported in plasma from patients with hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant disorder due to mutations in the endoglin gene [64]. We then measured by qRT-PCR hsa-miR-214 and hsa-miR-370 levels in cells and EVs from endoglin-expressing WM-164 cells (Fig. 14.7). Levels of hsa-miR-370 were similar in control and endoglin-expressing WM-164 cells, whereas the expression of hsa-miR-214 showed a non-significant increased trend in endoglin-positive WM-164 cells compared to controls (Fig. 14.7, left panels). In EVs from endoglin-transduced WM-164 cells, the levels of hsa-miR-370 displayed a significant reduction, while those of hsa-miR-214 showed a significant increase compared to mock-transduced WM-164 cells (Fig. 14.7, right panels). These results suggest that endoglin expression in melanoma involves the dysregulation of hsa-miR-214 and hsa-miR-370, which in turn could modulate melanoma progression.
In this work, we demonstrate a correlation between endoglin expression and tumor malignancy in primary melanoma and cultured melanoma cell lines. We have also deepened into the underlying endoglin-dependent molecular mechanisms, mainly focusing on the role of microRNAs in this process. Besides its physiological role in angiogenesis, endoglin has also emerged as a promising therapeutic target in recent years since endoglin expression has been reported either in tumor vessels or neoplasm in tumor cells, including melanoma, renal cell carcinoma (RCC), leukemias, certain subtypes of sarcomas, and breast, ovarian, endometrial, and prostate cancer. The role of endoglin in tumor cells depends on the cellular context. In this regard, and in line with our results obtained in melanoma, endoglin would be promoting tumor development and progression, playing an important role in oncogenic signalling (Fig. 14.8); whereas in other cases it has been associated with tumor suppression [4, 15, 82, 83].

In melanoma, endoglin has been pointed out to be essential for tumor plasticity, playing a key role in the interplay between TGF-β and BMP signalling pathways. Accordingly, endoglin downregulation hinders anchorage-independent growth and invasiveness and abrogates tumor growth in preclinical models of melanoma [8]. Moreover, experiments with shRNA against endoglin have shown to significantly reduce proliferation, survival and migration of melanoma cells [26, 30]. Recently, the therapeutic efficacy of a fusion protein containing endoglin single-chain variable fragment and IP10 (Endoglin-scFv/IP10) has been demonstrated. Indeed, this fusion protein inhibited proliferation and angiogenesis, while stimulating apoptosis within melanoma tissue [25]. In this context, our results further support the hypothesis that endoglin mediates malignant melanocyte transformation in WM-164, as the levels of the well-known melanoma markers PMEL and TYR2 increase upon endoglin overexpression. Furthermore, an increased trend of hsa-mir-214 levels is observed from cells (left panels) or their derived EVs (right panels) are shown (n = 4 per condition). The miRNA expression levels are displayed relative to WM-164 cells transduced with the empty vector. *p < 0.05; **p < 0.01 by two-tailed student’s t-test.
in endoglin-transduced melanoma cells. Interestingly, hsa-mir-214 dysregulation has been widely described in several tumors, including melanoma.

Cancer-derived extracellular vesicles, including EVs, can target different cell types in the tumor microenvironment modulating tumor growth and metastasis [84–86]. Of note, cellular endoglin expression significantly regulates both hsa-mir-214 and hsa-mir-370 in EVs, of which endoglin is also a component. Thus, compared to EVs from control cells, endoglin overexpressing cells show reduced levels of hsa-mir-370 while increased content of hsa-mir-214 in EVs. We hypothesize that these dysregulated microRNAs in EVs may play a relevant role in tumor development and metastasis (Fig. 14.8). For example, the reduction of hsa-mir-370 levels in EVs from endoglin-expressing melanoma cells could favour the process of neo-angiogenesis, which is necessary for tumor growth. This can be achieved because endoglin is negatively regulated by hsa-mir-370 [63], and endoglin is highly expressed by actively proliferating endothelial cells of the tumor vasculature [7]. Consequently, EVs from the primary melanoma tumors carrying lower levels of hsa-mir-370 would favour migration, proliferation, differentiation and adhesion of endothelial cells. Given the reported role of hsa-mir-214 in tumor progression [65, 66], increased levels of hsa-mir-214 in EVs from melanoma cells may act in a paracrine manner once taken up by neighbour melanocyte cells, thereby transforming them and contributing to tumor growth and development (Fig. 14.8). The EVs-mediated targeting of hsa-mir-370 and hsa-mir-214 may not be limited to neoangiogenic vessels or melanocytes, as an effect on additional non-cancer cells from the tumor environment is expected as well [87]. Apart from malignant cells, non-cancerous cells, including adipocytes, endothelial cells of tumor vessels, lymphocytes, tumor-associated macrophages (TAMs), and cancer-associated fibroblasts (CAFs), as well as
molecules produced and released by them, constitute the tumor microenvironment [88, 89]. Active and mutual interactions, through a paracrine signalling or circulatory and lymphatic systems, between tumor cells and the tumor microenvironment have been described to play decisive roles in tumor initiation, development and progression, metastasis, and response to therapies [90, 91]. Consequently, the tumor environment has received increased attention in the recent cancer literature [92, 93]. For instance, melanoma-associated fibroblasts (MAFs) have been described to have a role in melanoma progression, therapy resistance and immunosurveillance [94–96]. Moreover, a variety of immune cells, i.e., T and B lymphocytes, macrophages, neutrophils, dendritic and natural killer cells support the growth and invasiveness of melanoma cells, using multiple mechanisms. Among them, it is remarkable the downregulation in T lymphocytes of anti-apoptotic proteins, including Bcl-2, caused by melanoma-derived EVs containing miRNAs, such as hsa-mir-690 [97, 98]. A recent study has shown that hsa-mir-125b-5p transferred by cutaneous melanoma-derived EVs induces a tumor-promoting TAM phenotype in macrophages [99]. A role for EVs carrying hsa-mir-370 or hsa-mir-214 on malignant progression has been outlined. Breast cancer cells-secreted EVs with hsa-mir-370-3p cargo can aggravate breast cancer through downregulation of CYLD tumor suppressor in fibroblasts concomitantly with activation of the NF-κB signaling pathway, thereby promoting the tumor cell functions [100]. Interestingly, expression of endoglin, a target of hsa-mir-370, in CAFs regulates invasion and stimulates colorectal cancer metastasis [101]. Also, by sponging hsa-mir-370-3p, the circular RNA (circRNA) circ_0020710 can promote melanoma cell proliferation, migration and invasion in vitro, as well as tumor growth in vivo through the upregulated expression of the CXCL12 [102], a chemokine known to regulate melanoma metastasis to distant sites [103]. In the case of hsa-mir-214, its downregulation in CAFs contributes to migration and invasion of gastric cancer cells through induction of epithelial-mesenchymal transition (EMT) [104]. Accordingly, hsa-mir-214-3p has been proposed as a novel therapeutic target in pancreatic CAFs and human pancreatic stellate cells (hPSCs), as its inhibition led to inhibition of TGF-β-induced differentiation of pancreatic CAFs and reduced expression of myofibroblast markers during the differentiation of hPSCs to myofibroblasts [105]. Furthermore, a role of tumor-secreted miR-214 in the conversion of CD4+ T cells into immune-suppressive regulatory T cells, promoting tumor immune escape has been described [106]. Future independent studies remain to be performed to better understand the functional impact of the endoglin-induced dysregulated microRNAs in melanoma cells and their microenvironment, as well as the possible mechanisms involved.

Along with the hsa-mir-214 and hsa-mir-370 cargos, EVs derived from endoglin-enriched melanoma cells, also contain the protein endoglin, in agreement with previous reports in EVs from endoglin-expressing endothelial cells or primary hepatic stellate cells [49–51]. Although endoglin+ EVs have been proposed as biomarkers for metastatic breast cancer [10], the putative functional role of this endoglin cargo in cancer remains to be elucidated. It is well established that endoglin specifically binds integrins [22, 37, 46] and tumor cell-derived EVs contain integrins involved the generation of pre-metastatic niches in specific tissues promoting organ-specific metastases of several types of cancer including melanoma [47, 84, 107]. Accordingly, it is tempting to speculate that by interacting with integrins, endoglin could be involved in these malignant processes. Further investigations are needed to better understand the role of endoglin in melanoma development.

**Statements and Declarations**

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Disclosure of Interests All authors declare they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent for participation and publication was obtained from all individual participants included in the study.

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Increase in Frequency of Myeloid-Derived Suppressor Cells in the Bone Marrow of Myeloproliferative Neoplasm: Potential Implications in Myelofibrosis

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Abstract

The Philadelphia-negative myeloproliferative neoplasms (MPNs), defined as clonal disorders of the hematopoietic stem cells, are characterized by the proliferation of mature myeloid cells in the bone marrow and a chronic inflammatory status impacting the initiation, progression, and symptomatology of the malignancies. There are three main entities defined as essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF), and genetically classified by JAK2V617F, CALR, or MPL mutations. In MPNs, due to the overproduction of inflammatory cytokines by the neoplastic cells and non-transformed immune cells, chronic inflammation may provoke the generation and expansion of myeloid-derived suppressors cells (MDSCs) that highly influence the
adaptive immune response. Although peripheral blood MDSC levels are elevated, their frequency in the bone marrow of MPNs patients is not well elucidated yet. Our results indicated increased levels of total (T)-MDSCs (CD33+HLA-DR−/low) and polymorphonuclear (PMN)-MDSCs (CD33+/HLA-DRlow/CD15+/CD14−) in the bone marrow and peripheral blood of all three types of MPNs malignancies. However, these bone marrow MDSCs-increased frequencies did not correlate with the clinical parameters, such as hepatomegaly, leukocytes, hemoglobin, or platelet levels, or with JAK2 and CALR mutations. Besides, bone marrow MDSCs, from ET, PV, and PMF patients, exhibited immunosuppressive function, determined as T-cell proliferation inhibition. Notably, the highest T-MDSCs and PMN-MDSC levels were found in PMF samples, and the increased MDSCs frequency strongly correlated with the degree of myelofibrosis. Thus, these data together indicate that the immunosuppressive MDSCs population is increased in the bone marrow of MPNs patients and may be implicated in generating a fibrotic microenvironment.

**Keywords**

MPNs · Bone-marrow · MDSCs · Immunosuppression · Fibrosis · And TGF-β1

**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| CALR | Calreticulin |
| DC | Dendritic cells |
| ET | Essential thrombocytopenia |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| HD | Healthy donors |
| HSC | Hematopoietic stem cell |
| HU | Hydroxyurea |
| IL | Interleukin |
| JAK | Janus Kinase |
| LDH | Lactate dehydrogenase |
| MDSCs | Myeloid-derived suppressor cells |
| M-MDSCs | Monocytic myeloid-derived suppressor cells |
| MNCs | Mononuclear cells |
| MPL | Thrombopoietin receptor |
| MPNs | Myeloproliferative neoplasms |
| NK | Natural Killer |
| PMF | Primary myelofibrosis |
| PMN-MDSCs | Polymorphonuclear myeloid-derived suppressor cells |
| PV | Polycythemia vera |
| SE | Sedimentation |
| STAT | Signal transducer and activators of transcription |
| TGF-β1 | Transforming growth factor-β1 |
| T-MDSCS | Total myeloid-derived suppressors cells |
| TNF-α | Tumor necrosis factor-α |
| WBC | White blood cells |
| WHO | World Health Organization |

### 15.1 Introduction

BCR-ABL1/Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) are clonal hematopoietic disorders driven by somatic mutation in Janus Kinase (JAK)2, Calreticulin (CALR), or thrombopoietin receptor (MPL) genes. There are three main MPNs entities: Essential thrombocythemia (ET), mainly characterized by a megakaryocyte expansion and increased platelet count; polycythemia vera (PV) typically exhibits an increased erythrocytes mass along with leukocytosis and thrombocytosis, which is consistent with the panmyelosis detected in the bone marrow of the patients; and primary myelofibrosis (PMF), mainly characterized by an abnormal proliferation of myeloid cells, extramedullary hematopoiesis, and a variable grade of reticulin/collagen bone marrow deposition due to myelofibrosis [1]. Besides, the ET and PV incidences have a similar rate of 1–2 cases per 100,000 person-years in the United States. Meanwhile, PMF seems more infrequent,
with 0.3 per 100,000 person-years incidences [2].

Even though MPNs, with an aberrantly increased number of mature cells, in the first stages are displayed as an indolent disorder, for example, ET and PV mainly exhibit asymptomatic peripheral cytoses; all the MPNs possess the potential to evolve to end-stage myelofibrosis and bone marrow failure or to develop acute leukemia [3].

Under normal conditions, healthy persons exhibit an inflammatory cascade regulated by a fine-tuning interplay of neuro-hormonal factors and cytokines with cellular activities and responses. Dysregulation of this system is a hallmark feature of the MPNs. At the same time, MPNs are characterized by dysregulated inflammatory responses that drive the evolution of malignancies [4]. Inflammation has been demonstrated to contribute to the development, progression, symptomatic burden, and prognosis of MPNs, which is characterized by elevated levels of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [5, 6].

Namely, polyclonal myeloid cells such as granulocytes, macrophages, and dendritic cells are identified as the primary inflammatory cytokines source in MPNs. Their abnormal activation may lead to disease progression and therapy resistance [7]. All MPNs are associated with different degrees of BM fibrosis, while the severity of myelofibrosis is directly proportional to the growth factor and proinflammatory cytokine production [8]. In this sense, transforming growth factor-β1 (TGF-β1), one of the hallmarks of cancer inflammation, is identified as part of the cytokine signature for fibroblast proliferation and activation and the development of bone marrow fibrosis [9, 10].

Interestingly, all the MPNs sub-groups evolve from genetic mutations within pluripotent stem cell populations in the bone marrow that also accumulate during the development of the disease. JAK2 V617F was the first described mutation inherent to MPNs, with 96% in PV, 50% in ET, and 50% in PMF [4, 11]. The constitutive activation of JAK2 V617F provokes hyperactivation of the signal transducer and activators of transcription (STAT) transcription factors with the results of exacerbated myeloid growth and expansion [12]. Moreover, JAK-STAT signaling induces the expression of various critical mediators of cancer and inflammation, such as immunomodulatory cytokines and growth factors, which further cause a positive autofeedback via the JAK/STAT pathway, thus perpetuating the malignant cellular phenotype [4, 13]. The other two primary MPN driver mutations, CALR and MPL, also constitutively activate JAK2/STAT signaling to promote, in this way, the development of MPNs [14].

Consequently, in most of these, the transformed hematopoietic stem progenitor cell clone express mutations in the constitutively activated JAK2 and downstream signaling that further drives malignant hematopoiesis. Moreover, the dysfunctional cytokine production in MPNs, including overproduction of TGF-β1, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-3, and IL-6, could lead to the accumulation of immature myeloid cells such as myeloid-derived suppressor cells (MDSCs) [15, 16]. MDSCs seem to be one of the major obstacles to displaying natural antitumor immunity. They have been demonstrated to be abnormally elevated in inflammatory-associated diseases such as infection and diabetes [17, 18]. Specifically, MDSCs and their immunosuppressive activity have been related to the initiation and progression of cancer associated with chronic inflammation [19]. These immunosuppressive cells are mainly recognized as CD33⁺HLA-DR⁻/low with high inhibition capabilities on T cell proliferation and activation [20]. MDSCs exert their T-cell suppression by producing soluble factors involved in oxidative stress, such as reactive oxygen species, nitric oxide, and increased expression of inducible nitric oxide synthase. Also, they express several cytokines with immunosuppressive activities, such as IL-10 and TGF-β1 [16, 20]. Furthermore, MDSCs also demonstrate CD11b positivity, and by the expression of CD15 or CD14, they could be marked as polymorphonuclear (PMN-
MDSCs, expressing CD15) and monocytic (MDSCs, expressing CD14) [21]. In cancer, because of pathological signals produced by the tumor, MDSCs accumulate in bone marrow and spleen, wherefrom they migrate into peripheral blood and peripheral tissues, including tumors [22]. Moreover, PMN-MDSCs contribute to tumor growth and chemoresistance, as well as the resistance to immune checkpoint inhibitor therapy in melanoma patients through cytokine production and immunosuppression [23]. Although the cellular and molecular mechanisms of MDSC generation and expansion are an increased subject of investigation, it is well understood that these immunosuppressive cells arise in inflammatory conditions during an emergency myelopoiesis, namely when there is a growing demand for myeloid cells [24]. Under pathological conditions, such as infection or cancer, bone marrow emergency myelopoiesis responds to the increased production of inflammatory factors, such as GM-CSF, IL-6, and chemokines. In addition, these factors contribute to the generation and expansion of immunosuppressive MDSCs that collaborate in preventing excessive tissue damage due to an exacerbated immune response [16, 25]. Mainly, the MDSCs number is expanded as immature cells in the bone marrow. After that, they migrate to circulation, where terminal differentiation is blocked and converted into functional MDSCs [21]. Also, chronic inflammation may stimulate extramedullary myelopoiesis and promote the spleen and peripheral lymphoid organs’ MDSCs production, thus increasing the number of these cells in the resolution of the inflammation [26]. Besides, MDSCs have the potential to migrate to different peripheral organs and differentiate towards dendritic cells (DC), neutrophils, or macrophages to support immune cell functions [27]. T-cell lymphocyte (T-cell) inhibition is the hallmark of the functional features of MDSCs; At the same time, they may also inhibit DC and natural killer (NK) cells with lesser potency [20]. In this way, MDSCs contribute to generating an immune-tolerant tumor microenvironment associated with a poor cancer prognosis and tumor burden [28].

The bone marrow niche is a complex and specialized microenvironment essential for hematopoietic stem cell (HSC) functionality. This niche mainly comprises two related components, the cellular, and non-cellula entities, that are part of three principal compartments: the endosteal niche, which is composed of bone-associated metabolisms, such as osteocytes, that coordinate the activity of the two other cellular members; the osteoblasts and osteoclasts cells [29]; the perivascular niche that includes sinusoidal endothelium and arterioles, transition zone vessels, mesenchymal stroma/stem cells, and reticular cells; and the third compartment, the extracellular matrix (ECM), which as non-cellular domain provides a physical structure for bone marrow niche integrity. The ECM is formed by several structural proteins, mainly collagens I, II, III, IV, and X, laminin, reticulin, and fibronectin, among others. As well as ECM-embedded growth factors, cytokines, and chemokines [30]. All these compartments exhibit a finely coordinated interplay for HSC survival and proportioned a dynamic adaptation to external threats and ensuring HSC fate [2].

In MPNs, the bone marrow niche homeostasis seems to be disrupted, which highly contributes to the proliferation/expansion, and survival of clonal mutated HSC [2], therefore the switch of healthy bone marrow to a dysfunctional one with profound alterations within the bone marrow niches is implicated in the physiopathological mechanisms of MPNs that culminate in the development of inflammatory and profibrotic environment bone marrow [31]. Bone marrow fibrosis, or myelofibrosis, is generated by excessive deposition of reticulin and collagen fibers, which also allows evaluating and scoring bone marrow fibrosis by the hematopathologist based on extracellular matrix fibers density [32]. In MPNs, myelofibrosis represents PMF or arises from pre-existing ET or PV patients [9]. One of the hallmarks of myelofibrosis is elevated peripheral blood levels of proinflammatory cytokines [33]. Concerning bone marrow fibrosis, increased production of TGF-β in the bone marrow of MPNs has been described, mainly
produced by megakaryocytes and monocytes [33]. In particular, TGF-β contributes to the development of myelofibrosis and hematopoietic niche disruption in MPNs by regulating mesenchymal stromal cell differentiation towards myofibroblastic features [33]. In addition, monocyte-derived fibrocytes and endothelial cells-derived mesenchymal stromal cells may increase the number of fibroblastic cells in the bone marrow of MPNs patients [34]. Moreover, in the advanced stage of myelofibrosis, the bone marrow of MPNs patients develops osteosclerosis, a pathological bone modification due to a gradual loss of the marrow giving rise to a replacement by collagen fibers and bone trabeculae rich in activated myofibroblasts, which generate new bone shaping and bone volume growth. Thereby, these pathological events produced increased bone density and abnormal hardening that affects normal bone marrow activities [31].

Besides participating in creating fibrotic bone marrow, TGF-β1 regulates MDSC generation, expansion, and function. For instance, in combination with GM-CSF, this factor induces the MDSC in vitro with effective inhibition of T-cell proliferation. It may also induce T-regulatory cells, which together further promote an immunosuppressive tumor microenvironment [35, 36].

Although a previous study identified the increased frequency of CD11b+ , CD14−, and CD33+ cells MDSCs in the peripheral blood of MPNs patients [37], the levels of MDSCs in the bone marrow of MPNs patients remained unexplored. In this study, we have identified the frequency of total (T)MDSCs and the CD15+PMN-MDSCs in the bone marrow and peripheral blood of patients with ET, PV, and PMF and correlated levels of clinical and laboratory parameters. Furthermore, we analyzed the immunosuppressive ability of the cells by their capacity to inhibit T-cell proliferation and their correlation with MPNs bone marrow fibrosis. Our findings might have an important implication for understanding the patient immune status and mechanisms behind disease progression and myelofibrosis.

15.2 Methods

Patients and samples: Sixty-four MPNs de novo patients (ET 17; PV 29; and PMF 18) and 5 healthy donors (HD) were recruited for bone marrow. Thirty-one (ET 11; PV 11; and PMF 9) and 11 HD were recruited for peripheral blood analyses of MDSCs. All patients were newly diagnosed according to the World Health Organization (WHO) criteria at the Clinic for Hematology, Clinical Center of Serbia. Bone marrow (n = 5) and peripheral blood (n = 11) was obtained from age-matched healthy donors (HD) at the Institute of Blood Transfusion and Hemobiology, Military Medical Academy, Serbia. Patients and donors signed the informed consent form in accordance with the Declaration of Helsinki. All patients were previously untreated, and data including age, gender, laboratory findings (white blood cells (WBC) and platelets count, hemoglobin, LDH levels, SE rate), size of the liver and spleen, grade of bone marrow fibrosis, JAK2 and CALR mutation status, the requirement of hydroxyurea (HU) treatment; were collected at the diagnosis.

Flow cytometry analysis of MDSCs: Iliac crest bone marrow and peripheral blood samples of healthy voluntary donors and patients were collected in tubes with ethylenediaminetetraacetic acid (EDTA). Mononuclear cells (MNCs) were separated by density gradient centrifugation with a lymphocyte separation medium (LSM, Capricorn Scientific GmbH, Germany). Briefly, each sample was diluted 1:1 with Ca2+/Mg2+—free PBS and then layered gently on the top of ten ml lymphocyte separation medium. After centrifugation (400 g, 30 min, 20 °C), the mononuclear cells’ interfaces were collected and washed twice with room-temperature PBS. Viable cells were determined using the trypan blue technique (Gibco, Thermo Fisher Scientific, MA, USA). After that, 1 × 10^6 MNCs from MPN patients and HD were freshly obtained and subjected to immunophenotype analysis. 0.5 × 10^5 cells in PBS were stained using anti-CD33-PE, anti-HLADR-APC, anti-CD14-FITC, and anti-CD15-
PerCP antibodies (BioLegend, CA, USA) for 20 min at 4 °C. Then, cells were washed three times with room temperature PBS and subjected to flow cytometry analysis (BD FACS-Calibur, Becton Dickinson, Heidelberg, Germany). Generally, 50,000 events per sample were acquired, and the results were evaluated by the NovoExpress software (Agilent, CA, USA).

MDSCs depletion and T cell proliferation analysis: To deplete MDSC from HD and patients’ samples, 5 x 10^6 MNCs in RPMI medium (three samples from each group) were incubated for 15 min at 4 °C with anti-CD33 magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) as described previously [38]. After washing with PBS, labeled cells were run through LS columns and placed on a MACS separator (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s instructions. The non-trapped cells were then collected for T-cell proliferation analyses. At the same time, a small number of cells were stained with anti-CD33 and anti-HLA-DR antibodies for flow cytometry analysis, to ensure that MDSCs were successfully depleted. Further, CD33-depleted MNC samples and MNC samples without depletion from the same donor were subjected to comparative analysis of T cell proliferation by CFSE proliferation assay. Briefly, 10^5 cells per ml were incubated with 1 μM CFSE (Sigma–Aldrich, St. Louis, MO, USA) for 10 min at RT. A drop of fetal bovine serum was added to stop the labeling reaction and to complete the staining procedure; then, cells were washed three times with room temperature PBS. The proliferation of T-cells was stimulated with 20 mg of αCD3/CD28 micro-beads (Invitrogen, UK), and cells were left in culture with RPMI supplemented with 10% FCS (Sigma–Aldrich, St. Louis, MO, USA). After three culture days, cells were washed twice with PBS and fixed with 2% formaldehyde/PBS until they were subjected to FACS analysis of the CFSE proliferation assay. CFSE dilution was estimated using the unstained and zero-time cells (related to the cells just after CFSE staining treated with Mitomycin C (40 μg/ml for 30 min). The proliferation of the cells was calculated based on CFSE dilution by FCS EXPRESS 5 software (De Novo Software, Los Angeles, CA, USA) [39].

Azan trichrome staining: MPNs patients’ trephine biopsy samples were subjected to azan trichrome staining for visualization of collagen fibrils, and fibrosis assessment. Sections were deparaffinized and rehydrated by using xylene, descending alcohol (100, 96, 70%), and distilled water for 15 min each. Then, sections were immersed in Azocarmine B solution for 30 min, washed, and left for 1 min in 1% Anile-alcohol solution for differentiation. The reaction was stopped by adding the acid alcohol, following the treatment of the sample with 5% Phosphotungstic acid for 60 min. Further, sections were stained using Azan reagent for the next 60 min and dehydrated in ascending series of alcohol solution (70, 96, and 100%) each for 2 min. Finally, sections were cleared by washing twice in xylene, and a Mount medium was added. As a result of the staining procedure, nuclei were red, while fibers were stained blue. All reagents were provided by Sigma–Aldrich, St. Louis, MO, USA. Samples were analyzed by using the microscope (Olympus Provis AX70). Myelofibrosis was graded according to the WHO 2016 grading system that included four grades (MF 0–3) [7, 40/37].

Statistical analysis: Statistical analyses were made with the student’s t-test or ANOVA test. The Spearman test was used to analyze the correlation between the study groups. Data are given as means ± SEM, and p-values < 0.05 are considered to indicate significant differences. All statistical analyses were conducted using the GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA).
15.3 Results

15.3.1 Myeloproliferative Neoplasm Patients’ Clinical Data

This study included 64 MPNs de novo patients. The clinical data of these enrolled patients at diagnosis are shown in Table 15.1. Out of the 64 patients, 17 were classified as having ET, were classified as having PV, and 18 were classified as having PMF. 55% of the ET patients were positive for the JAK2 V617F mutation, 100% PV harbor JAK2 V617F mutation, and 67% of PMF patients carrying JAK2 V617F. Meanwhile, 21% of ET and 22% of PMF patients carry CARL mutations. The median age of the ET patients, comprising five males and 12 females, was 56 years (range: 28–80 years); while for PV patients, 15 males and 14 females, was 60 years (range: 32–85 years); for PMF patients, 14 males and four females, the median age was 62 (range: 36–87 years) at the time of diagnosis. Meanwhile, the grade of fibrosis was analyzed by the Azan trichrome staining at the time of diagnosis by staining collagen fibers on bone marrow biopsies. 72% of ET patients present grade 1 fibrosis, PV patients exhibit 55% of grade 1, 31% of grade 2, and 3.4% of grade 3, and PMF patients present 5 of 6% of grade 1, 22.2% of grade 2, and 11.1% of grade 3 of fibrosis.

15.3.2 The Frequency of T-MDSCs and PMN-MDSCs is Elevated in the Bone Marrow of MPNs Patients

To identify and evaluate the increased frequency of MDSC in the bone marrow, the mononuclear isolated cells of MPNs patients, described in Table 15.1, and HD samples (N = 5, four males and one female median age 36 (range: 25–41 years) were subjected to immunophenotyping as described by Bronte et al. ‘s report [20]. The T-MDSCs immunophenotyping is defined as CD33⁺/HLA-DR<sup>low</sup> myeloid cells, while PMN-MDSCs were identified as CD33⁺/HLA-DR<sup>low</sup> / CD15⁺/CD14⁻ cells. The quantity of T-MDSCs and PMN-MDSCs in the bone marrow samples freshly obtained from HD and ET, PV, and PMF patients at diagnosis, was evaluated using flow cytometry. The gating strategy and representative results for one HD and one MPNs patient from each group are presented in Fig. 15.1a. The results indicated that the percentages of bone marrow T-MDSCs and PMN-MDSCs were significantly elevated in all of the MPNs samples compared to HD (ET 35.17 ± 9.4%, p = 0.04 for T-MDSCs and 40.4 ± 15.1%, p = 0.038 for PMN-MDSCs; PV 44.05 ± 8.33%, p = 0.01 for T-MDSCs and 39.23 ± 11.8%, p = 0.031 for PMN-MDSCs versus HD 25.36 ± 12.7% T-MDSCs and 18.8 ± 10.4% PMN-MDSCs), most prominently in PMF (55.37 ± 12.38% versus HD 25.36 ± 12.7%, p = 0.004 for T-MDSCs and 54.58 ± 14.3% versus HD 18.8 ± 10.4%, p = 0.008 for PMN-MDSCs) (Fig. 15.1b and c).

Noticeably, the analysis of the correlation between the clinical data and the number of MDSCs indicated that the elevated bone marrow-associated MDSCs frequencies did not correlate with age or hepatomegaly, leukocytes, hemoglobin, or platelet levels. Furthermore, no correlation was observed between MDSCs and JAK2/CALR status (data not shown).

15.3.3 The Frequency of T-MDSCs and PMN-MDSCs is Increased in the Peripheral Blood of MPNs Patients

Previous studies demonstrated significantly high circulating CD11b⁺, CD33⁺, and CD14⁻ MDSCs levels in the peripheral blood of MPNs patients [37]. Consequently, in this study, we investigated the presence of T-MDSCs and the PMN-MDSCs in the freshly obtained peripheral blood samples of 31 MPNs patients at diagnosis and 11 HD.
Consistent with the previous observation, the levels of T-MDSCs were elevated in the peripheral blood of MPNs patients compared to healthy donors. Significantly higher levels of T-MDSCs were noticed in the peripheral blood of PV and PMF patients compared to HD (PV, 19.7 ± 6.23, p = 0.008; PMF, 35.53 ± 9.84, p = 0.004 versus HD 7.44 ± 3.8), while the levels of circulating T-MDSCs in ET were moderately increased but did not reach statistical significance (Fig. 15.2a). Moreover, significant differences in levels of circulating PMN-MDSCs between HD and all MPNs entities were detected (PMF 14.78 ± 5.03%, p = 0.01; PV 9.36 ± 3.54%, p = 0.026 and ET 6.15 ± 2.75, p = 0.023 versus HD 3.83 ± 1.4) (Fig. 15.2b). Further analysis of the absolute number of MDSCs (MDSCs/μl) calculated using the WBC count revealed similar results. As shown in Fig. 15.2c and d, ET patients have a high number of MDSCs relative to normal (468.04/μl, p = 0.004 for T-MDSCs and 348.78/μl, p = 0.0008 for PMN-MDSCs), while PV (544.67/μl, p = 0.0029 for T-MDSCs and 417.56/μl, p < 0.0001 for PMN-MDSCs) and PMF (718.53/μl, p < 0.0001 for PMN-MDSCs) patients have significantly higher numbers compared to HD (331.24/μl, p = for T-MDSCs and 229.12/μl for PMN-MDSCs). The quantity of M-MDSCs (CD33+/HLA-DRlow/CD14+/CD15−) in peripheral blood and bone marrow samples of MPNs patients did not show significant differences related to HD (data not shown).

| Table 15.1 MPN patients’ clinical characteristic |
|-----------------------------------------------|
|                                 | ET   | PV   | PMF  |
| No of patients                  | 17   | 29   | 18   |
| Age at diagnosis (range)        | 56 (28–80) | 60 (32–85) | 62 (36–87) |
| Males/females                   | 5/12 | 15/14| 14/4 |
| WBC, 10^9/l (range)             | 9.6 (6.5–13.7) | 12 (28.5–5.3) | 11.7 (1.8–19.6) |
| Hemoglobin, g/l (range)         | 143 (117–161) | 156 (126–184) | 138 (96–164) |
| Platelets, 10^9/l (range)       | 827 (348–1417) | 604.9 (81–1354) | 715.7 (265–1397) |
| LDH, U/l (range)                | 435 (99–647) | 525 (340–964) | 553 (187–1934) |
| SE, mm/h (range)                | 18 (2–72) | 7 (2–28) | 19 (2–118) |
| Enlarged liver (AP diameter larger than 15 cm), (%) | 5 | 10 | 16 |
| Enlarged spleen (AP diameter larger than 13 cm), (%) | 11 | 37 | 44 |
| JAK2 V617F mutation, (%)        | 55   | 100  | 67   |
| Calreticulin mutation, (%)      | 21   | 0    | 22   |

Bone marrow fibrosis

|                                 | ET   | PV   | PMF  |
| Grade 1, (%)                    | 72   | 55   | 56   |
| Grade 2, (%)                    | 0    | 31   | 22.2 |
| Grade 3, (%)                    | 0    | 3.4  | 11.1 |
| HU treatment, (%)               | 66   | 46   | 44   |
| Number of deaths, (%)           | 0    | 3.4  | 11   |

The main clinical parameter of essential thrombocytopenia (N=17), polycythemia vera (N= 29), and primary myelofibrosis (N=18) patients are indicated. All patients were newly diagnosed according to the World Health Organization (WHO) criteria. Abbreviations: ET-essential thrombocytosis, PV-polycythemia vera, PMF-primary myelofibrosis, WBC- white blood cells, LDH-lactate dehydrogenase, SE-sedimentation rate, AP-antero posterior, HU-hydroxyurea.
15.3.4 MPNs-Related MDSCs Exhibited Immunosuppressive Capabilities

To verify whether the increased MDSCs populations in MPNs patients may exhibit an immunosuppressive function, we tested their capacity to inhibit T-cell proliferation by an MDSCs depletion assay. After reducing the MDSCs number in the peripheral blood mononuclear cells fraction (from 52.7 to 20.91%) by a CD33 microbeads system (Fig. 15.3a), a T-cell proliferation assay was performed. The analysis was performed by selecting three patients’ samples per group for each ET, PV, and PMF MPN sub-malignancies under a complete number of MNC cells or subjected to MDSCs depletion. CFSE-labeled T-cells were stimulated with anti-CD3 and CD8 antibodies for 72 h, and the dilution of CFSE fluorescence was determined by flow cytometry. The results, shown in Fig. 15.3b and c, indicated that the MPNs-isolated MNC fractions, under MDSCs depletion, exhibited a significant improvement in T-cell proliferation rate. The ET patients’ samples were significantly increased to 95.2% with MDSCs depletion compared to 41.05% in the sample without depletion (p = 0.001). Similar results were obtained in PV (87.2% versus 46.1%, p = 0.005) and PMF patients’ samples (88.09% versus 46.5%, p = 0.005). Therefore, these results suggested that the elevated MDSCs frequency in the peripheral blood of MPNs patients effectively displayed immunosuppressive T-cell activities.

Fig. 15.1 Significantly elevated levels of total MDSCs and PMN-MDSCs in the bone marrow of patients with MPN. a Flow cytometry analysis was performed with gates set on CD33 HLA-DRlow (total MDSCs), and within this population, the fraction of cells expressing CD15 and negative for CD14 was determined (CD33 HLA-DRlowCD15CD14 PMN MDSC).

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15.3.5 Fibrosis in the Bone Marrow of MPNs Patients

To evaluate fibrosis of BM, paraffin-embedded bone marrow samples from ET, PV, and PMF patients were stained with Azan Trichrome. According to collagen deposition marked with blue, we determined the degree of bone marrow fibrosis according to what is defined by Barbui et al. [40] and Arber et al. [41]. As is shown in Fig. 15.4a, a low-grade collagen deposition, with only perivascular staining, was named grade 0; moderate, short, and thin collagen fibers were named grade 1. Meanwhile, a high level with tick collagen fibers forming a network was marked as grade 2/3.

Remarkable, high levels of T-MDSCs frequency were found in MPNs patients with bone marrow fibrosis grades 2/3 (mean 72.64 two, \( p = 0.00.004 \)), compared to patients without fibrosis (45.52%). In comparison, patients with grades two had moderately increased levels of T-MDSCs (48.9%, \( p = 0.19 \)) (Fig. 15.4b). Moreover, the PMN-MDSCs population was significantly increased in all patients with bone marrow fibrosis (grade 1, 41.6%, \( p = 0.048 \); grades 2/3, 59.7%, \( p = 0.00,041 \)) related to patients without fibrosis (30.07%) (Fig. 15.4c). Meanwhile, the correlation analyses indicated that the MDSCs levels were correlated with the degree of bone marrow fibrosis. A significant correlation was found between the percentage of T-MDSCs (Pearson \( r = 0.3448; \) \( p = 0.0107 \)) as well as PMN-MDSCs (Pearson \( r = 0.398; \) \( p = 0.006 \)) and levels of bone marrow fibrosis (\( n = 46 \) patients).
15.3.6 TGF-β1 Induces MDSC from Bone Marrow Mononuclear Cells

Previous results marked that TGF-β1 expression is increased in the bone marrow of MPNs patients and is one of the primary mediators of bone marrow fibrosis in patients with PMF [42, 43]. Next, we investigate the effect of TGF-β1 combined with GM-CSF and stem cell factor (SCF), on the induction of T-MDSCs from MNC of HD and MPNs system. The flow cytometry analyses of CD33 and HLA-DR positive populations revealed that 3- and 6-day-long treatment with these factors significantly increased levels of T-MDSCs (Fig. 15.5a and b). These results suggest that elevated TGF-β1, responsible for significant bone marrow fibrosis, could also lead to the induction of bone marrow MDSCs in patients with MPNs.

15.4 Discussion

Despite that MPNs are defined as clonal hematopoietic stem cell disorders characterized by the proliferation of mature myeloid cells in the bone marrow [2], in the last years, MDSCs, a group of cells with immature characteristics and immunosuppressive functions, have been implicated in regulating inflammation in MPNs [44, 45]. Specifically, MPNs exhibit a dysregulation in the function and number of immune cells, such as increased frequency of monocytes and macrophages and expansion of MDSCs [46]. This study mainly focuses on analyzing MDSCs in the bone marrow of all three subtypes of MPNs patients, ET, PV, and PMF. Although total and PMN-MDSCs frequency is elevated in MPNs at diagnosis, with the highest number in PMF, no correlation between clinical parameters and
MDSCs frequency was found. Meanwhile, the level of M-MDSCs did not increase in MPNs compared to healthy donors. Moreover, when the frequency of peripheral blood circulating MDSCs was determined, an increased number of T-MDSCs was also established. Consistently, PMN-MDSCs were found to be increased in peripheral blood MNC fractions of MPNs patients. Interestingly, PMF patients exhibit the highest MDSCs frequency independent of their bone marrow or peripheral blood source. Furthermore, and as expected, MPNs-associated MDSCs demonstrated efficacy in inhibiting T-cell activation and proliferation. Therefore, the results of this study showed that immunosuppressive MDSCs frequency is elevated in the bone marrow of MPNs patients, mainly represented by the PMN-MDSCs subgroup, and the elevated peripheral blood MDSCs levels confirm what was reported by Wang et al. [37]. Similarly, as reported in this study, MDSCs levels did not correlate with the presence of JAK2V617F or Calreticulin allele burden, white blood cells, hemoglobin levels, platelet counts, and splenomegaly.

Besides, it is now recognized that MDSCs may represent an inflammatory link with the inhibition of antitumor T-cell immunity in hematologic neoplasms [14]. Moreover, since MDSCs have immunosuppressive and tumor-promoting activities, their increased levels, mainly PMN-MDSCs, observed in high-grade cancers correlate with a poor prognosis, including diffuse large B-cell lymphoma, Hodgkin lymphoma, and chronic lymphocytic leukemia [47]. Although the result of this study suggested that the highest levels of MDSCs correlate with fibrosis grades 2/3, whether MDSCs levels correlate with poor prognosis in MPNs needs to be further confirmed.
Interestingly, in chronic lymphocytic leukemia, a sub-MPNs group carrying the Philadelphia chromosome, the treatment with the tyrosine kinase inhibitors imatinib and dasatinib [48], two BCR- and ABL-mediated oncogenic signaling inhibitors, efficiently depleted MDSCs along with IL-10 and Arginase-1 biomarkers reduction [49]. In this sense, MPN chemotherapy considers the use of cytoreductive hydroxyurea as the first line of treatment and the JAK2 inhibitor ruxolitinib [50, 51]. However, no studies have addressed the potential of MPNs therapies to decrease MDSCs levels. In this case, preliminary analysis performed in our laboratory indicated the potential for hydroxyurea to reduce MDSCs levels in MPNs patients, but this has been performed in a limited number of patients that does not permit obtaining conclusive results.

Besides acute myeloid leukemia, myelodysplastic syndrome and chronic lymphocytic leukemia also have an increased level of MDSCs in the bone marrow and peripheral blood. In all these myeloid malignancies, MDSCs highly contribute to systemic and bone marrow immunosuppression contributing to transformed myeloid cell expansion [52]. Furthermore, acute myeloid leukemia-elevated MDSC frequency in the bone marrow is associated with a significantly shorter overall survival and poor prognosis. In contrast, a low number of MDSC-like blasts indicates leukemia-free survival of AML patients [53]. Also, the neoplastic myeloid cell transformation may contribute to the establishment of chronic inflammation that further promotes selective clonal expansion over normal hematopoietic cells [54]. In MPNs, increased
inflammation may be generated, in one way, by transformed clones themselves and by influencing nonmutant bystander immune cells to adopt proinflammatory phenotypes. In particular, in MPNs, prominent chronic inflammation can be caused by the JAK2V617F mutant cells, for example, due to their aberrant inflammatory cytokine production, creating thus the conditions to activate other cells to an inflammatory stage [55]. So, it is possible to speculate that the inflammatory scenario created by neoplastic cells, with increased production of proinflammatory cytokines, mimics an emergency myelopoiesis and makes a perfect condition for generating and expanding MDSC in the bone marrow and peripheral blood of MPN patients. Interestingly, chronic lymphocytic leukemia-derived MDSC seems to be composed of nonmutated cells and cells harboring driver oncprotein BCR/ABL protein [56]. Nevertheless, whether MPN-derived MDSCs harbor the driver JAK2, CALR, and MLP mutations needs further investigation.

Notably, ET and PV are generally less aggressive forms of malignancies. Both may progress to myelofibrosis, with worsening marrow fibrosis mainly characterized by the accumulation of extracellular fibers in the stroma of the bone marrow [57]. The evaluation of MPNs-associated bone marrow fibrosis, according to a semiquantitative 0–3 scale grading system for collagen deposition [58], and MDSCs levels in the bone marrow of MPNs patients indicated a correlation between the degree of tissue fibrosis and total and PMN-MDSCs frequency. MPNs are mainly characterized or classified according to the bone marrow morphology and the quantity of marrow fibrosis, along with clinical, laboratory, cytogenetic, and molecular features [2]. Bone marrow fibrosis is one of the distinctive features of PMF, accompanied by massive splenomegaly and peripheral splenomegaly [59].

Moreover, all MPNs may exhibit a potential to evolve to end-stage myelofibrosis, which causes either bone marrow failure or progression to secondary acute myeloid leukemia [3, 60]. Although the primary cause of PMF is not well elucidated, several pathogenic mechanisms of myelofibrosis have been described in MPNs. Predominantly, bone marrow fibrosis may result from a nonclonal proliferation and hyperactivity of fibroblast due to elevated inflammatory cytokines and growth factors expressed mainly by clonally expanded megakaryocytes [61]. Also, bone marrow fibrosis may be understood as a complex interaction among megakaryocytes, monocytes/macrophages, endothelial cells, and fibroblasts [62]. For instance, due to exacerbating bone marrow megakaryocyte activity, elevated amounts of TGF-β1 are produced [63]. In this scenario, this cytokine is a potent inducer of fibrosis with the capacity to control the bone marrow mesenchymal stromal cell's fate towards fibroblasts/myofibroblasts along with increasing collagen I, collagen III, and fibronectin deposition [9, 34, 64–66]. Also, monocytes may contribute to MPNs’ pathogenesis of bone marrow fibrosis via TGF-β1 production. This process implicates a monocyte differentiation towards clonal-derived fibrocytes co-expressing hematopoietic and stromal cell markers [2, 34].

In turn, MDSCs also secreted copious amounts of TGF-β1 [62], which may contribute to the pathogenesis of bone marrow fibrosis. As well, elevated bone marrow TGF-b levels may induce MDSCs expansion and immunosuppressive activities [48], generating a positive loop that may consolidate inflammation and myelofibrosis in MPNs. Nevertheless, the exact contribution of MDSCs to bone marrow fibrosis needs to be elucidated in further investigations.

Despite the immunosuppressive MDSCs functions, some investigations demonstrated its role in the induction of fibrosis. For instance, adoptive MDSCs transfer in a mouse xenograft cancer model contributes to the generation of fibrotic tumor stroma and cancer-associated fibroblast activation [67]. Also, PMN-MDSCs may participate in the production of cardiac fibrosis along with myofibroblast activation [68]. However, none of these investigations have linked MDSCs and TGF-β with fibrosis.

In summary, this study demonstrated increased frequency levels of MDSCs in the bone marrow of MPNs patients. Although increased MDSC levels did not correlate with MPNs
clinical parameters or JAK2 or CALR mutations, the results indicated a correlation of MDSC-increased bone marrow frequency with the intensity of myelofibrosis. These results may picture the potential for considering MDSCs as a prognostic marker in MPNs. They may also represent a link between chronic inflammation and inhibition of anticancer T-cell immunity in MPNs [2].

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**Ethical Approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Approval was granted by the ethics committee of the Clinical Center of Serbia, Belgrade (decision number 4/1). This article does not contain any studies of the Clinical Center of Serbia, Belgrade (decision number 4/1). This article does not contain any studies of the Clinical Center of Serbia, Belgrade (decision number 4/1). This article does not contain any studies of the Clinical Center of Serbia, Belgrade (decision number 4/1). This article does not contain any studies of the Clinical Center of Serbia, Belgrade (decision number 4/1).

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The “Ins and Outs” of Prostate Specific Membrane Antigen (PSMA) as Specific Target in Prostate Cancer Therapy

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Abstract

Prostate-specific membrane antigen (PSMA) is expressed in epithelial cells of the prostate gland and is strongly upregulated in prostatic adenocarcinoma, with elevated expression correlating with metastasis, progression, and androgen independence. Because of its specificity, PSMA is a major target of prostate cancer therapy; however, detectable levels of PSMA are also found in other tissues, especially in salivary glands and kidney, generating bystander damage of these tissues. Antibody target therapy has been used with relative success in reducing tumor growth and prostate specific antigen (PSA) levels. However, since antibodies are highly stable in plasma, they have prolonged time in circulation and accumulate in organs with an affinity for antibodies such as bone marrow. For that reason, a second generation of PSMA targeted therapeutic agents has been developed. Small molecules and minibodies have had promising clinical trial results, but concerns about their specificity had arisen with side effects due to accumulation in salivary glands and kidneys. Herein we study the specificity of small molecules and minibodies that are currently being clinically tested. We observed a high affinity of these molecules for PSMA in prostate, kidney and salivary gland, suggesting that their effect is not prostate specific. The search for specific prostate target agents must continue so as to optimally treat patients with prostate cancer, while minimizing deleterious effects in other PSMA expressing tissues.
16.1 Introduction

Prostate cancer (PC) remains a highly prevalent disease, and one in 9 men will suffer from PC during their lifetime [1]. Although present with demographic differences, worldwide it is the second leading cause of cancer deaths in men. Often overlooked in mortality statistics, are the huge impacts on the quality of life for men and their partners from the various therapeutic modalities, especially for later stage disease. Approximately half of patients will be offered androgen deprivation therapy (ADT), radiotherapy, chemotherapy, and/or immunotherapy, for biochemically recurrent, locally advanced, or metastatic disease [2]. Most patients who start ADT live symptom-free for years, but must endure adverse effects including: physical, mental, sexual and metabolic [3]. More troubling, almost all men with metastatic PC progress from ADT-responsive to ADT-resistant end-stage disease with little hope of a cure. Finding a way of avoiding ADT while extending life would significantly improve their quality of life. More optimistically, the possibility of an all-out cure at all stages of disease could be within reach. The concept of targeted therapy has been known since Ehrlich imagined an antibody-based “magic bullet” that would deliver a toxin to cancer while sparing normal tissues [4]. However, over the past century, attempts at targeted therapy have largely failed. Yet the “magic bullet” for PC is evolving from promise to reality due to the following scientific advances.

Prostate-specific Membrane Antigen (PSMA) specific targeting has been used for therapeutic and diagnostic purposes. PSMA, is highly expressed on the surface of PC cells and its expression increases with tumor grade and in
metastatic sites [5]. For this reason, antibodies, small molecules, and antibody-based constructs such as minibodies have been developed to target PSMA and carry radioactive isotope particles to identify and/or induce the death of PC cells [6]. PSMA was first detected in 1987 as an antigen of monoclonal antibodies developed through murine hybridomas against the human prostate adenocarcinoma cell line LNCaP [7]. The authors described the reactivity of these antibodies as “very narrow and limited to the surfaces of LNCaP cells only”. Then, they tested their novel antibodies against sections of 175 human tissue specimens and observed positive immunoreaction in the epithelial cells of PC and hypertrophic and normal prostate tissue, as well as 2 out of 11 kidneys, suggesting that PSMA could be a specific target for PC.

PSMA gene was cloned as 2.65-kb cDNA for a transcript that encodes a 750-amino acid protein [8], that was mapped in chromosome 11p13-p11.1 [9]. PSMA shares 97% amino acid identity with PSMAL (FOLH1B), that is thought to be a duplicated gene that maps in 11q14 [10]. PSMA is a type II transmembrane glycoprotein belonging to the M28 metallopeptidase family, which includes aminopeptidases and carboxypeptidases. These enzymes need divalent cations (preferentially Zn but can also use Mg or Ca) as enzymatic cofactors [11]. PSMA has a short (18 amino acid) N-terminal cytoplasmatic tail [12], followed by a single transmembrane helix (25 amino acids) and a large extracellular portion (706 amino acids). PSMA has several N-glycosylation sites [8], for a final molecular weight of 100 kDa. In its N-terminal domain, PSMA interacts with several cytoplasmic proteins to allow internalization and recycling.

PSMA catalytic function was firstly describe by Pinto et al. [13]. By isolating PSMA from LNCaP cells membranes, they found a glutamate carboxypeptidase that cleaves amino acids from the C-terminal of proteins and peptides by hydrolysis on different alternative substrates, including the nutrient folate (pteroylmonoglutamate), the neuropeptide N-acetyl-l-aspartyl-l-glutamate, and the chemotherapeutic, methotrexate [13]. The latter activity has been used to explain why PSMA expressing cancers are resistant to methotrexate treatment. Interestingly, PC cells that did not react to anti-PSMA antibodies, such as PC-3, DU-145, or TSU-Pr1, did not show folate or glutamate hydrolase activity. In brain, PSMA-like mRNA was described by Northern blot analysis [14] and its translation to a functional protein was later confirmed by immunoprecipitation and functional assays [15]. The brain-expressed protein demonstrated to have hydrolase activity catabolizing the neuropeptide N-acetylaspartylglutamate (NAAG). For those reasons PSMA is also known as Glutamate carboxypeptidase II (GCP2), N-acetylated-alpha-linked acidic dipeptidase (NAALADase), and Folate hydrolase I (FOLH1).

Although PSMA is known for being a marker of prostate cancer, it is also expressed in other tissues. In mouse models, PSMA (GCP2) has been described in brain, kidney, and salivary gland, but interestingly not in prostate [16]. Human tissues that express PSMA include brain (neurons, type II astrocytes and Schwann cells), kidney, salivary gland, liver, colon, testis, ovary, prostate, small intestine, and spleen [17, 18]. Early studies that developed antibodies against human PSMA described positive immunostaining in frozen sections of prostate acini, proximal tubules of kidney, and weak staining in duodenum. This tissue-restricted expression pattern suggests a role for PSMA in very specific biochemical processes in each tissue. Furthermore, contemporary imaging technologies in human patients, using positron emission tomography (PET) to visualize radiolabeled PSMA tracers $^{68}$Ga-PSMA or $^{18}$F-PSMA have also shown uptake in lacrimal gland, liver, spleen and gallbladder, and mild uptake in nasal and esophageal mucosa, vocal cords, trachea, and bronchi [19, 20]. Importantly, PSMA has been described in the vasculature of a variety of cancers [21]. Such as metastatic renal cell carcinoma [22], bladder cancers [23], glioblastoma multiforme [24], hepatocellular carcinoma, testicular-embryonal, neuroendocrine, colon, among others, which strongly suggests PSMA role in neovascularization process. Interestingly this neovascularation cancer-associated PSMA+ is not present in PC [25].
The development of radiotherapeutic anti-PSMA targeted antibodies and small molecules increased the relevance of diverse PSMA expression, since targeting organs that are different from PC could potentially cause severe off-target effects. PSMA-J591 antibody was the first antibody that specifically recognized the extracellular domain of PSMA, and it was used in combination with radio-emitter particles for specific PC targeting. While showing promising results in tumor growth, limitations due to its large size which prevents penetration in solid tumors, serum stability which maintains radioactive elements in circulation for long periods of time, and accumulation in tissues with affinity for antibodies (e.g. bone marrow), led to the search for new alternatives. Small molecules were then developed to address these limitations. Although targeting prostate PSMA, and with promising preliminary results, $^{124}$I-PSMA and $^{131}$I-PSMA still showed elevated uptake in salivary glands, kidney and liver [26] resulting in xerostomia (dry mouth), anemia and kidney dysfunction in clinical studies[27]. A newer generation of targeted agents has attempted to achieve a more selective prostatic PSMA targeting to decrease these adverse effects. As such, the small molecules PSMA-617 (vipivotide tetraxetan) and J519 antibody-based PSMA-minibodies have shown more promising results in clinical trials [28–30]. We tested these two specific PSMA target agents and tested against cell lines and tissues to evaluate their affinity and specificity. Our results show that both, PSMA-617 and a J591-based-minibody have a high affinity and specificity for PSMA. However, since they strongly interact with prostate cancer tissue, they similarly also interact with salivary gland and kidney. We hypothesize that the observed selectivity in clinical images of J591-based minibodies is due to the restricted localization of PSMA to the luminal surface of the cells, compared to small molecules like PSMA-617 which will diffuse into cells. Our results suggest that the adverse effects of treatment with PSMA-targeted agents will continue to be a problem in the near future due to the lack of prostate specificity of the currently used agents, and the search for prostate-specific targets must continue.

16.2 Methods

Patients: All procedures were performed under approval from the ethics board of the University of British Columbia (H19-02061) and were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable. All patients provided signed consent. We obtained tissue from submandibular salivary glands resected as part of radical mandibulectomy because of non-salivary pathology (4 Squamous cell carcinoma of tongue, 1 squamous cell carcinoma of sublingual mucosa, 1 squamous cell carcinoma of the tongue, 1 aggressive ameloblastoma of the jaw) from 7 patients (4 women, mean age 69 YO). Parotid non tumoral tissue was obtained from 4 patients that underwent partial parotidectomy as consequence of myxoid pleomorphic adenoma (3 women, mean age 56 YO). Non-tumoral kidney tissue was obtained from 4 radical nephrectomies as consequence of renal clear cell carcinoma (1 woman, mean age 66 YO). Since renal clear cell carcinoma tissue is distinctive from normal tissue, non-tumoral cortical and medullar kidney tissue was dissected out macroscopically and confirmed microscopically by an experienced pathologist (Dr. Eric Belanger). We obtained sections of prostate carcinoma in different grades from the genitourinary biobank of the Vancouver Prostate Centre (ethics approval No V09-0320).

Histology and Immunohistochemistry: All tissue sections were divided into two specimens, one fixed in Buffered formalin for 24 h to continue regular histological processing, and one specimen was snap frozen for cryosection. Snap freezing was performed by immersing the freshly obtained samples in liquid nitrogen, and subsequently embedding them in optimal cutting temperature compound (OCT). The tissue samples were stored at −80, and tissue slides were
created in a cryostat. 6-micron sections were mounted on poly-lysine coated glass slides, fixed in 95% ethanol for 2 min and used immediately. Formalin fixed samples were dehydrated in ascendent concentrations of alcohol (70, 80, 90, 95, and 100% x 3 for 1.5 h each) and cleared in xylene substitute (2 x 2 h), before paraffin infiltration (3 x 1.5 h) and embedding. 5-micron sections were created using a microtome (Leica), and mounted on poly-lysine coated glass slides. All samples were stained with hematoxylin-eosin following established protocols [31].

For immunodetection of PSMA, we used the following commercially available antibodies: rabbit monoclonal EPR6253 (Abcam, ab133579), mouse monoclonal 3E6 (Agilent, M3620); for frozen sections we used rabbit polyclonal (Sigma HPA010593), and mouse monoclonal (LifeSpan BioSciences LS-B3040) antibodies. After deparaffinization, the tissue slides were rehydrated and endogenous peroxidase was quenched by incubation with hydrogen peroxide 10%. Since salivary peroxidase and myeloperoxidase are abundantly secreted in saliva because of their antimicrobial properties [32], quenching endogenous peroxidases process was extended in salivary gland sections up to 4 changes of peroxide every 10 min in paraffin embedded sections, and up to 8 changes every 10 min in frozen sections. After washing with PBS, non-specific protein block was performed with 3% bovine serum albumin (BSA) in PBS for 30 min. Primary antibodies were incubated overnight at 4°C. After thorough washing with PBS, horseradish peroxidase conjugated secondary antibodies, anti-mouse (Abcam, ab205719) or anti-rabbit (Abcam, ab205718), were incubated for 90 min at 37°C. This antibody was then visualized with HRP-conjugated anti-mouse antibody and the immunoreaction was revealed by using a diaminobenzidine substrate kit (Vector).

For fluorescent visualization, the minibody was conjugated with Alexa fluor 488 (UBC Antibody Lab) and used in a 1/100 dilution in BSA 0.2 M arginine for immunofluorescence in cell culture and tissue sections. We incubated the minibody solution on the slides overnight at 4°C and nuclear staining was performed after washing with Hoechst 33342 (ThermoFischer). The slides were mounted by using a fluorescence mounting medium (Dako, S3023), and visualized under epifluorescence with the microscope and cameras described above.

Fluorescently Cy3 dye labelled vipivotide (PSMA-617) was prepared and provided by Dr. Jerome Lozada as described previously [33]. For histological visualization, after non-specific protein binding, a 0.01 mg/mL solution in 3% BSA was incubated for 1 h at room temperature. After washing with PBS, we performed nuclear staining with Hoechst 33342, slide mounting and visualization as described above.

Cell Culture: PCa cell lines: C4-2, and PC3, and Human Embryonic Kidney cell HEK293T were obtained from ATCC (Manassas, VA). LNCaP cell lines were cultured in Roswell Park Memorial Institute (RPMI, Invitrogen, Burlington, ON, Canada) 1640 media supplemented with 10%
fetal bovine serum (FBS, Invitrogen). HEK293T cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS. All cell lines were maintained at 37 °C and 5% CO2.

**PSMA Truncations and Point Mutations:** To generate the PSMA truncations, a pcDNA3.1 plasmid containing variant 3 PSMA from LNCaP n-terminally linked mCherry was used. Truncations were created from the 3’ end by PCR mutagenesis so that aside from the full-length molecule of 750 amino acids, truncations were made available at 740, 452, 316, and 177 amino acids. Glycosylation mutations of PSMA were generated by converting the glycosylation associated asparagine N-476A to alanine using site directed mutagenesis. Modified PSMA variants were investigated through the transfection of HEK293T cells using Lipofectamine™ LTX Reagent with PLUS™ Reagent (ThermoFisher Scientific) and flow cytometry analysis was performed two days after transfection.

**PSMA KO LNCaP Generation:** LNCaP cells (ATCC) were transiently transfected with a pool of lentiCRISPRv2 plasmids containing one of three sgRNAs which targeted exons 8, 13, and 16 of PSMA with 19 bp target regions (sgRNA (1): AGCCACGCCACGCTCTTGA, sgRNA (2): AGCCAGGTCCAACATTGTA, sgRNA (3): TTTTAGTATACCGTGCTC). Puromycin selection was used to enrich for transfected cells. PSMA KO cells were enriched by probing for PSMA using an AlexaFluor 488-conjugated anti-PSMA antibody (ThermoFisher Scientific, GCP-05) and sorting for the negative population in two successive rounds of FACS. The knockout was confirmed through flow cytometry, western blotting, and amplicon generation sequencing.

**Flow Cytometry:** Cells were lifted using EDTA, washed in PBS and incubated with the fluorescently labeled (described above) J591-based minibody or PSMA-617 on ice and protected from light for one hour in a HEPES buffer. Analysis was performed on a BD FACSCANTO II flow cytometer using 488 nm excitation.

### 16.3 Results

#### 16.3.1 PSMA Localization is Restricted to Secretory Poles of Specific Epithelial Cells

We started our observations of PSMA-targeted agents by using traditional immunohistochemistry with commercially available antibodies (Fig. 16.1). As expected, we observed staining for PSMA in the secretory pole of epithelial prostate cells, with reduced staining in other parts of the cells and negative staining in the surrounding connective tissue (Fig. 16.1b and c). As previously described, PSMA expression is associated with the degree of anaplasia of PC cells [5]. Consistently, we observed mild immunostaining in well-defined single layered acini (Fig. 16.1c), with increased staining in those acini showing anaplastic changes such as multiple cell layers, cell crowding and folding (Fig. 16.1d). Furthermore, in highly anaplastic acini, we observed PSMA not restricted to the secretory pole of the cells but compromising the whole cell surface and cytoplasm in the most anaplastic areas (Fig. 16.1d).

Xerostomia is a major side effect of PSMA-targeted radiotherapeutic small molecules and as such deserves a deeper appreciation of the details of salivary gland PSMA expression and targeted agent affinity. We separately analyzed the two major salivary glands: submandibular and parotid, due to their different secretory patterns that include different protein composition and viscosity [34, 35]. Submandibular is a mixed gland that generates a seromucous secretion, due to the presence of two well differentiated type of secretory acini, serous and mucous. While parotid is a predominantly serous gland, with minimum mucous component, and sublingual is preferentially a mucous gland. Serous acini are histologically described as rounded or elliptical clusters of 5–20 cells in a single section (Fig. 16.2a–d), which are characterized by their affinity for hematoxylin in routine H-E staining.
(basophils), and rounded nuclei located in the basal 1/3 of the cells. In their secretory pole, the cells have a great number of sub-micron diameter basophilic granules, which are loaded with their secretion content, mostly composed of enzymes such as amylase and proline rich proteins [36]. Mucous acini present low affinity for conventional H-E staining (Fig. 16.2b), thus they are observed as clear structures, in which the nuclei adopt a basal location, but instead of round they appear as ovoid or even lentil-shaped, with their major axis perpendicular to the major axis of the cells (Fig. 16.2b). Mucous acini secrete a viscous saliva with high concentration of mucins. In mixed glands, mucous acini usually present a serous crescent (Gianuzzi) around them (Fig. 16.2b). From the secretory acini, saliva is transported through a series of collecting tubes. The first part of this tubular system is the intercalated tube, composed of cubic simple epithelium (Fig. 16.2c), which continues with the striated duct and major collecting tubes (Fig. 16.2d), characterized by larger lumens, columnar epithelial cells with elongated nuclei. By using immunohistochemistry in formalin fixed paraffin embedded submandibular glands, we observed that PSMA localized predominantly in serous acini (Fig. 16.2e and f), with the immuno-staining being restricted to the secretory pole of the cells, including cell border and part of the cytoplasm, which is consistent with the known membrane localization and internalization...
Fig. 16.2 PSMA localization in Submandibular gland. 

a Low magnification image of histological section, in which the variety of histological elements can be observed, such as serous and mucous acini, collecting ducts. b Higher magnification of mucous acini (asterisk) and serous acini (circle). Mucous acini are characterized by a clear cytoplasm under regular histological staining and lentil-shaped nuclei in the basal pole of the cells (arrowhead). Serous crescents (arrows) are observed around mucous acini (asterisks). c Intercalated ducts (arrows) are composed by cuboidal epithelium with round-shaped nuclei (arrowheads) and are observed in vicinity of serous acini (circles). d Striated ducts are formed by a columnar epithelium (arrows) and bigger lumen (asterisk). e Low magnification of PSMA immunohistochemistry on submandibular gland. Positive PSMA detection is observed in acini and smaller ducts (arrowheads), but negative staining in striated and major collecting ducts (arrows). f Higher magnification showing negative staining in mucous acini (asterisks) but positive staining in serous acini (circles). g Positive PSMA staining of intercalated duct (arrows), serous acini (circles) and serous crescents (arrowheads). h Negative PSMA detection in striated ducts (arrows). Bar = 100 mM. Staining A-D = hematoxylin–eosin
of PSMA [37]. However mucous acini showed no staining (Fig. 16.2f). The intercalated duct showed intense immunostaining also restricted to the luminal membrane with minimal cytoplasmatic staining (Fig. 16.2g), while no PSMA detection in striated ducts (Fig. 16.2h) or major collecting tubes (Fig. 16.2e).

Consistent with the description for submandibular glands, and using the same technical approach, we observed PSMA in serous acini and intercalated duct of parotid glands (Fig. 16.3a–c), but not in striated ducts (Fig. 16.3d) or major collecting tubes. Of note, detection of PSMA in salivary gland from women showed similar intensity to those obtained from men (Fig. 16.3), suggesting no sex differences in PSMA expression in non-prostatic tissue and raises the question of how the androgen receptor axis modulates PC PSMA expression.

Kidney is another major organ where PSMA-targeting has been described to generate deleterious effects [27]. Kidney’s functional unit, the nephron, is composed of the glomeruli, where blood is filtered, followed by a series of tubules. The proximal convoluted tube, the largest of the renal tubules, is composed of a tall cubic epithelium, with rounded nuclei and a brush border or microvilli that increases the contact surface with the secreted fluid and extends towards most of the lumen. The cytoplasm of these cells is highly eosinophilic and thus stains strongly pink in H-E sections (Fig. 16.4). The Loop of Henle consists of simple squamous epithelium and is located mostly in the renal medulla. The distal

![Fig. 16.3](image-url) PSMA localization in parotid gland. a Low magnification of immunohistochemical staining of PSMA, with positive staining for acinar structures (arrowheads) but negative in collecting ducts in a predominantly adipose connective matrix. b Higher magnification image showing positive PSMA immunostaining of serous acini, preferentially in the lumina border of secretory cells (arrowheads). c Positive PSMA-immune detection on intercalated ducts (arrows), restricted preferentially to the luminal pole of the cells. d Negative PSMA-detection on striated duct (arrows), surrounded by positive stained acini (arrowheads). Bar = 100 mM
convoluted tube is shorter, with a wider lumen and composed of cubic epithelium that is less eosinophilic with less microvilli than the proximal convoluted tubule. In formalin fixed paraffin embedded kidney sections, we observed PSMA immunostaining restricted to the luminal border of the proximal convoluted tubule are PSMA negative, as well as distal convoluted tubes (arrowheads), while proximal convoluted tubes stain positive for PSMA (arrows). d–f Higher magnification images showing positive staining for PSMA in the luminal surface of proximal convoluted tubules (arrows), while glomeruli (asterisks) and distal convoluted tubes (arrowheads) remain negative. Bar = 100 microns. Staining A-B = Hematoxylin–eosin.

**Fig. 16.4** PSMA localization in human kidney. a Low magnification histological section of kidney cortex, showing glomeruli (asterisks) and multiple tubules sections. b High magnification mage of kidney cortex, where segments of proximal convoluted tubes (arrows), distal convoluted tubes (arrowheads), and Henle loop (red arrows) are shown. c Low magnification image of PSMA immunodetection in kidney cortex. Glomeruli (asterisk) (Fig. 16.4c and d) and absent in the distal convoluted tubule (Fig. 16.4f) Loop of Henle and collecting tubules of the medulla. The staining of the lumen of the proximal convoluted tubules, suggests that it is present in the microvilli membrane, where PSMA may have a role in the glutamate interchange.
16.3.2 Small Molecule PSMA-617 is not Selective for Prostate PSMA and Can Recognize Salivary and Kidney PSMA

PSMA antibody based theranostics has faced many challenges as the large molecular size of antibodies does not allow them to easily penetrate solid tumors, limiting their therapeutic capabilities. Furthermore, antibodies are very stable in plasma so that they remain a long time in circulation, increasing the exposure time of non-cancer tissues, especially those with some PSMA expression or affinity for antibodies [6]. For these reasons, small molecules have been developed as therapeutic carriers of radioemitters or cytotoxic drugs. PSMA-617 is a small molecule that has been charged with $^{177}$Lu and used in several clinical trials that successfully reduced PC progression markers. To evaluate the tissue specificity of PSMA-617 we used a Cy3 fluorescent conjugated version and characterized its PSMA binding in tissue frozen sections. As expected, we observed that PC tissue stains strongly with PSMA-617 (Fig. 16.5a and b). In salivary gland we observed a strong staining on serous acini (Fig. 16.5c and d), but negligible staining in mucous acini (arrowheads in Fig. 16.5d), while in kidney, we observed PSMA-617 strongly stained the proximal convoluted tubules (arrows in Fig. 16.5e) but not distal convoluted tubules (arrowheads in Fig. 16.5e) or collecting tubes (Fig. 16.5f). These results confirm that small molecules have no specificity for prostate PSMA and suggests that they might actually lead to an increase in adverse effects under prolonged treatment.

16.3.3 J591-Based Minibody Binds a Portion of the Extracellular Domain of PSMA by the Region of Binding of PSMA-617

In the search for selective prostate PSMA ligands, antibody-based targeting agents were the first developed agents to be studied in clinical trials with relative success [37]. J591 is a highly sensitive antibody that targets the extracellular domain of PSMA. It has been proposed that J591 is selective for prostatic PSMA with minimal affinity for PSMA in other tissues, possible due to a much lower PSMA expression [38]. To overcome the limitations of antibodies, such as reduced penetration in solid tumors, prolonged time of clearance, and deleterious effects on tissues with affinity for antibodies, a J591 based minibody conjugated with $^{89}$Zr ($^{89}$Zr-IAB2M) was engineered and used in clinical trials [29]. We tested the specificity of our J591-based minibody construct (VPC-PSMA-101).

By using flow cytometry on HEK293T cells transfected with different PSMA truncations, we determined that the VPC-PSMA-101 (J591-based minibody) binds in the extracellular domain between amino acids 316 and 346, consistent with the epitope proposed previously [39] and with a loss of binding that occurs when PSMA is truncated at amino acid 316 (Fig. 16.6a–c).

It has been previously proposed that mutation of asparagine N-476A, that harbors the glycosylation site, would significantly decrease the binding of J591 antibody [40]. However, the described domain that J591 binds to does not include this residue. In fact, it is on the opposite side of the molecule. While trying to confirm if this mutation would be critical in the epitope recognition of J591-based minibody, we tested a PSMA mutant. We found no changes in binding with alanine mutation to these glycosylation sites. (Fig. 16.6d). We are unaware of any other reports attempting to validate this early observation. We conclude that differences in glycosylation of these residues is not a potential mechanism of differential J591 affinity for PSMA presented on PC versus normal tissues.

16.3.4 J591-Based Minibody is not Specific for Prostatic PSMA and Can React with PSMA of Other Tissues

We next tested the selectivity and specificity of the J591-based minibody, with imaging on human cells and tissues. By immunofluorescence
labeling of LNCaP (PSMA+) and PC3 (PSMA-) prostate cancer cell lines, we confirmed that J591-based minibody selectively stains LNCaP but not PC3 cells or PSMA-KO cells (Fig. 16.7a and b). By using direct immunofluorescence (Fig. 16.7c) and immunohistochemistry (Fig. 16.7d), we characterized the expression of PSMA in PC frozen tissue sections. When used on sections of kidney and salivary gland, the J591 minibody shows positive staining of the luminal part of serous acini and intercalated tubes (Fig. 16.7e) and the proximal convoluted tubule in kidney (Fig. 16.7f), while other parts of tissues remained negative to PSMA staining, as expected. These results confirm the selectivity of J591 minibody for PSMA but suggests that J591 minibody does not have specific affinity for prostate PSMA compared to other tissues. Differences seen may be related to the pharmacokinetics of the minibody absorption, distribution, and clearance or the higher expression of PSMA in prostate adenocarcinoma.
16.3.5 PSMA KO Model Confirms J591-Based Minibody Specificity

To confirm the specificity of the J591-based minibody, we developed a KO LNCaP cell line. Through flow cytometry we determined that J591-based minibody showed significantly higher affinity (~100-fold) for wt LNCaP compared to KO cells (Fig. 16.8). This difference was much higher than that observed for PSMA 617 (Fig. 16.8b) suggesting that J591-based minibody is a more selective carrier of therapeutic agents than PSMA 617. These results confirm that the reactivity of the J591-based minibody is specific to PSMA, and not to other proteins that could be differentially expressed in LNCaP cells compared to PC3.

16.4 Discussion

Selective tumor targeting has been a key objective in cancer therapy over the years. In this context, PSMA selectivity is critical in the future of prostate cancer therapy, due to its high expression in PC and low expression in most other tissues. Non-prostate PSMA expression is a major cause of adverse ‘bystander’ effects of specific targeted therapy. Antibodies, small molecules and minibodies have been used as carriers of radioisotope particles for theranostic or therapeutics, that have been successful in limiting tumor progression, but generating a variety of adverse effects due to this non-specific targeting. Our analysis of PSMA targeting agents that aim to selectively target PC, shows that...
antibodies, small molecules and minibodies and have high affinity and specificity for PSMA, but there is no evidence of specific PC PSMA selectivity. Thus, the promising results observed in clinical imaging of antibodies and minibodies are due mostly to the specific location of PSMA in the lumen of glands and organs, where the exposure to big organic molecules such as antibodies is low, but small molecules that can diffuse into these surfaces also generate more severe adverse effects.

One example of this phenomenon is with the small molecule PSMA-617 conjugated to the beta-emitter 177Lutetium. Studies with this molecule showed positive results, with PSA decline after treatment [41, 42]. However, reports of adverse effects such as anemia, neutropenia, thrombocytopenia, xerostomia significantly increased after...
repeated treatments (rechallenge) [43]. Alpha emitters such as $^{225}\text{Ac}$ would be more specific in cancer treatment because of its lower penetration depth (1 mm) which would more selectively affect the tissues that express PSMA, with expected lower negative bystander impacts. $^{255}\text{Ac}$-PSMA-617 has been used in clinical trials, where it has demonstrated to be effective in PSA reduction [44]; however maintaining elevated rates of side effects including xerostomia (63% of patients) [45] proposed to be due to the presence of PSMA expression in salivary glands. As we show in this manuscript, although restricted to serous acini and intercalated duct, PSMA is expressed in all major salivary glands that are responsible for 90% of total saliva production [46]. Furthermore, the lowest expression (and thus lower effect of PSMA agents) is presumably in the mucous glands (sublingual) that only contributes 5% of total saliva, while the highest expression is in the predominantly serous gland parotid which is responsible for only 25% of saliva during resting conditions but 60% of salivary secretion under stimulation, thus increasing the perception of dry mouth when eating.

Antibody based agents with high affinity for PSMA have been used as guidance systems, mostly conjugated with radioactive isotopes $^{177}\text{Lu}$, $^{255}\text{Ac}$, $^{277}\text{Th}$, $^{90}\text{Y}$, $^{85}\text{Zr}$, $^{89}\text{Zr}$ or $^{131}\text{I}$, for both diagnostic imaging and therapeutic purposes [6]. Successful imaging trials have demonstrated a high sensitivity of PSMA-J591 antibody conjugated with $^{85}\text{Zr}$ for PET/CT-scanning to detect PC metastases to skeletal and soft tissues [29]. The subsequent development of J591-based PSMA minibody (IAB2M) aimed to overcome the adverse effects of antibody-based therapy (prolonged time in circulation and accumulation in tissues with antibody affinity such as bone marrow. Phase I and Phase II clinical trials for the $^{89}\text{Zr}$-desferroxiamine conjugated IAB2M have shown in PET/CT a similar performance to $^{85}\text{Zr}$-J591 imaging [29]. As the therapeutic community waits for the results of phase III clinical trials, we tested the specificity of a similar minibody for PC PSMA. Our results, demonstrating affinity of J591-based minibodies for salivary and kidney, suggests that if J591-based minibodies are secreted and/or present in those tissues, xerostomia and some degree of renal dysfunction are expected. However, the higher molecular weight than the small molecule 617 should give the minibody constructs significant advantages by reducing its glomerular filtration and salivary secretion.

Our observations, show that while PSMA detection is strongly associated to the luminal border of salivary glands, prostate and kidney, the protein localization also extends to the cytoplasm of epithelial cells. This could be due to a process of PSMA internalization that was first observed in LNCaP cell cultures by laser scanning confocal microscopy, where the fluorescence labelling was observed in the cytoplasm of LNCaP cells 2 h after antibody exposure [36].
Immunogold labeling and TEM observation revealed that the internalization process of PSMA occurs in 60 nm clathrin coated vesicles [36]. Further analysis revealed that the intracellular domain of PSMA interacts with the globular N-terminal domain of clathrins, which is not common in clathrin binding proteins [47]. This interaction is through a specific sequence (MWNLL) in its amino terminal segment has been described as critical to PSMA internalization [48]. Additionally, the cytoplasmic tail of PSMA interacts with alpha-adaptin, a protein that is part of the clathrin adaptor protein-2 complex (AP-2). Interestingly, it was demonstrated that in human immortalized endothelial cells, one fifth of PSMA internalization occurs also in caveolae, where it colocalizes with transferrin, and PSMA is still internalized after blocking clathrin coated vesicles [49]. This internalization process could be a potential advantage in the development of PSMA-ligands with cytotoxic elements that will be internalized and may exert their function intracellularly, which represents an opportunity to develop active agents different from currently studied alpha or beta emitters.

In conclusion, our work confirms the selectivity and specificity of J591-based minibody and PSMA-617 in binding PSMA expressing cells and tissues, with high affinity for prostate cancer, but also with affinity for salivary gland and kidney. This non-prostate cancer tissue affinity causes the secondary effects that will limit their usefulness as targeting-agents in patients. We believe that the prostate PSMA selectivity of J591 antibodies and minibodies, suggested by clinical imaging, are due mostly to the restricted location of PSMA in areas with low exposure to antibodies. Thus, the search for specific agents that selectively bind PSMA will eventually provide more effective diagnostics and therapeutics for prostate cancer patients with minimum adverse effects, thus ultimately improving their quality of life.

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Abstract

Transforming growth factor-beta1 (TGF-β1) regulates a plethora of cell-intrinsic processes that modulate tumor progression in a context-dependent manner. Thus, although TGF-β acts as a tumor suppressor in the early stages of tumorigenesis, in late stages, this factor promotes tumor progression and metastasis. In addition, TGF-β also impinges on the tumor microenvironment by modulating the immune system. In this aspect, TGF-β exhibits a potent immunosuppressive effect, which allows both cancer cells to escape from immune surveillance and confers resistance to immunotherapy. While TGF-β inhibits the activation and antitumoral functions of T-cell lymphocytes, dendritic cells, and natural killer cells, it promotes the generation of T-regulatory cells and myeloid-derived suppressor cells, which hinder antitumoral T-cell activities. Moreover, TGF-β promotes tumor-associated macrophages and neutrophils polarization from M1 into M2 and N1 to N2, respectively. Altogether, these effects contribute to the generation of an immunosuppressive tumor microenvironment and support tumor promotion. This review aims to analyze the relevant evidence on the complex role of TGF-β in cancer immunology, the current outcomes of combined immunotherapies, and the anti-TGF-β therapies that may improve the success of current and new oncotherapies.

Keywords

TGF-β1 · T-cell lymphocyte · Immunosuppression · Cancer · Immunotherapy
17.1 Introduction

The tumor microenvironment (TME) is fundamental to creating favorable conditions for the cancer cells to proliferate and evolve into malignant stages, where they acquire the capabilities to metastasize or colonize distant organs. In this complex tumor evolution process, the transforming growth factor β-1 (TGF-β1), hereafter TGF-β, is implicated in almost all stages of cancer progression, such as cell proliferation, migration, invasion, survival, angiogenesis, and metastasis. However, the effect of TGF-β on tumor development is dual. While TGF-β displays tumor-suppressive activities at early stages, in cancer cells in the late stage of malignancy, TGF-β promotes the progression of cancer cells into a more proliferative and aggressive state [1, 2]. In addition to the cell-intrinsic effect of TGF-β, this factor also modulates other cellular components of the TME. TGF-β is able to modulate both innate and adaptive immune cell responses, which generates a complex immunosuppressive network that favors cancer cells escape from immune surveillance [2–5]. Therefore, targeting TGF-β effects both on cancer cells and immune cells is a rational therapeutic strategy. Indeed, many efforts and therapeutic strategies aim to target TGF-β to recover or potentiate the antitumor function of the immune system.

In this review, we analyze the complex role of TGF-β in cancer immunology. We also aim to convey insights on the main aspects of TGF-β in tumor progression, focusing primarily on regulating the TME-associated immune system. Furthermore, we address current outcomes of immunotherapies against TGF-β, which open novel immunotherapies approaches to improve both the success of new and existing oncotherapies and the quality of life of cancer patients.
17.2 Transforming Growth Factor-β 1 Signaling

TGF-β is one of the three TGF-β isoforms described in mammals (β1, β2, and β3). Although these three subtypes share a high level of sequence homology, their functions could be divergent [6]. Bioactive TGF-β is involved in several biological processes associated with tumor growth and progression, such as cell differentiation, proliferation, apoptosis, and angiogenesis, and possesses an immune response suppression activity, as well as in establishing a protumoral stroma, and finally promotes cancer cell metastasis [2, 3, 5]. The transcription control of the TGF-β gene also reflects its link with tumorigenesis. Despite that the TGF-β gene promoter lacks a classic TATA box, its transcriptional activation is promoted by several cis-elements and oncogenes and is repressed by tumor suppressor factors [7]. TGF-β is initially produced as a precursor of 75 kDa that can be cleaved by a furin-type convertase, generating the small latent complex (SLC) composed of the immature TGF-β and the latency-associated peptide (LAP). Thereafter, the SLC can bind the latent TGF-β binding protein, forming a second protein complex that remains covalently associated with the extracellular matrix (ECM) until TGF-β is released [8–10]. Interestingly in immune cells, the TGF-β complex can bind to the cell surface membrane via crosslinking with the leucine-rich repeat-containing protein 32/33. This interaction allows the crosstalk between immune cells by directly presenting pro-TGF-β to the receptor on the target cells [11]. The bioavailability of TGF-β depends on diverse releasing mechanisms such as plasmin or metalloproteinases dependent proteolytic cleavage, integrin-mediated activation, oxidative stress, and an acidic ECM microenvironment [8, 12, 13]. After the release of TGF-β from the ECM, it binds to its specific cell surface receptors to lead to a diverse signal transduction cascade implicated in the biological functions of the target cells [9]. TGF-β binds and activates the type-II receptor kinase (TβR2) that subsequently interacts and activates the type-I receptor kinase (TβR1), giving rise to the generation of a hexameric signaling complex able to activate the canonical and non-canonical TGF-β intracellular signaling [14, 15]. Moreover, the TβR3, also called betaglycan, with high affinity, may interact with TGF-β promoting its receptor binding and intracellular signaling [9, 16].

The canonical signaling comprises the activated receptor complex that recruits and activates by phosphorylation the inner plasmatic membrane-associated mediators Smad2 and Smad3. Their phosphorylation promotes the translocation of these proteins from the membrane to the cytosol, enabling interaction with the common partner Smad4. This heteromeric effector complex is translocated to the nucleus, which in association with other transcription factors, regulates TGF-β responsive gene targets [14, 17, 18]. Interestingly, among the target genes, TGF-β-Smad pathways induce a negative feedback response mediated by the expression of Smad7, an inhibitory Smad, which by competing with Smad2,3 prevent their binding to TβR1, therefore blocking the canonical intracellular transduction pathway [19, 20].

The non-canonical TGF-β signaling, also termed non-Smad pathways, involves the activation and crosstalk of several intracellular pathways such as mitogen-activated protein kinases (MAPK) ERK1,2, JNK, and p38, phosphoinositide 3-kinase (PI3K)/AKT1,2 and mammalian target of rapamycin (mTOR), nuclear factor κB (NF-κB), cyclooxygenase-2 (COX-2) and prostaglandins, the small GTPase proteins Ras, and Rho family of GTPases. Nevertheless, these pathways are not TGF-β signaling specific as they can be regulated by other signaling pathways, such as receptor tyrosine kinases [3, 21, 22]. Altogether, the diversity of TGF-β signaling explains the ability of TGF-β to influence a broad variety of molecular, cellular, cellular, and physiological activities.
17.3 Transforming Growth Factor-β Role in Cancer

TGF-β acts as a tumor suppressor in normal epithelial cells in the early stage of carcinoma by inhibiting proliferation and inducing apoptosis in premalignant epithelial cells [2, 23]. In particular, the tumor suppressor features of TGF-β are reflected in its capacity to inhibit cell proliferation in epithelial cells by regulating the expression of cyclin-dependent kinase (CDK) inhibitors, including p15Ink4b and p21Cip1. These proteins inhibit cyclinD-CDK4/6 and cyclinE/A-CDK2 complexes while inducing p16ink4a and p19ARF expression in order to promote a hyperphosphorylated state of the tumor-suppressor retinoblastoma (Rb). Hyperphosphorylated Rb forms a complex with E2F transcription factors, inhibiting its capacity to promote cell proliferation. E2F transcription factors regulate several essential genes implicated in the progression through the G1 to the S-phase of the cell cycle and DNA replication, thereby, their inhibition contributes to cell arrest [24–27]. In addition, TGF-β also inhibits cell cycle progression by repressing the expression of the transcription factor c-Myc and its DNA binding and promoting cell differentiation (Id1, 2, and 3) [28–30]. This factor also regulates cell survival and apoptosis. On the one hand, TGF-β induces cell death by upregulation of the death-associated protein kinase (DAP-kinase) expression, which in turn, promotes the association of TβR2 with the adaptor protein DAXX enabling the induction of JNK-mediated apoptosis. TGF-β also induces apoptosis by the so-called TGF-β signals (ARTS), which translocate from the mitochondria to the nucleus, thus inhibiting the X-linked inhibitor of apoptosis protein functions [31–33].

In contrast, in the late stage of carcinogenesis, TGF-β acquires protumoral properties as a result of multiple genetic and epigenetic modifications on the TGF-β signaling members that inhibit its tumor-suppressive functions. Indeed, TGF-β favors tumor growth, development, and metastasis [2, 3, 23, 29]. Among the major alteration of the TGF-β signaling in cancer, the downregulation of TGF-β receptors due to gene deletion, mutations or epigenetic silencing provokes a ligand compensatory overexpression, which leads to an increase in tumor aggressiveness [34]. For instance, TβR2 is epigenetically silenced by histone acetylation and DNA methylation; however, the expression of this receptor can be restored using 5-Aza-2'-deoxycytidine, an inhibitor of DNA methyltransferase, which also reestablishes the cell cycle arrest in esophageal squamous cell carcinoma [3, 35]. In addition, the SMAD4 loss by homozgyous deletion has been described in several cancer types including human pancreatic cancers, sporadic gastroenterological tumors, human skin squamous cell carcinomas, human cervical cancer, head and neck squamous cell carcinoma, and colorectal cancer [36]. The loss of function of SMAD4 is also associated with a germline mutation in the autosomal dominant familial juvenile polyposis syndrome, which results in benign noncancerous masses in the digestive tract [37]. These data support the notion that Smad4 exerts tumor suppression functions. In contrast, inactivation of the TGF-β signaling such as SMAD2-inactivating mutations has been described with very low frequency in several cancers, such as colorectal, lung, cervical, and hepatocellular carcinomas. Indeed, low levels of Smad2 mRNA correlate with better survival of patients with oral squamous cell carcinoma [38, 39]. Conversely, no SMAD6 and SMAD7, the inhibitory Smads, exhibit cancer-linked mutations. Moreover, an increased Smad6 expression correlates with better survival in squamous cell carcinoma patients, and high Smad7 expression reduces TGF-β signaling in hepatocellular carcinoma [39, 40].

TGF-β levels seem to correlate with the malignancy stage of cancer development, and it is a valuable factor for the diagnosis, prognosis, cancer biomarker, and therapeutic target [41–43]. Indeed, in melanoma, breast cancer, renal cancer, prostatic cancer, and pancreatic cancer, elevated plasma level of TGF-β is associated with the advanced stages of cancer, metastasis, and poor clinical outcome [44–46]. Moreover, TGF-β
Peripheral circulating levels are also elevated in multiple myeloma and non–Hodgkin lymphoma, and it is highly increased in high-grade and cutaneous T-cell lymphomas with a T-regulatory phenotype, among others [47 and references therein]. Although elevated TGF-β levels can be produced by cancer cells, tumor-infiltrating myeloid cells and local stroma cells can also contribute to its production, promoting tumor progression and metastasis via autocrine and paracrine mechanisms [2, 3].

Finally, the tumor promoter functions of TGF-β are not only limited to the pro-metastatic cancer cells, but it also acts on the stroma cells, such as cancer-associated fibroblasts and immune cells, as well as creates an immunosuppressive TME, which further favors tumor development and growth [2, 3, 5, 48].

17.4 Transforming Growth Factor-β and Cellular Immune System Interactions

The TME has considered a complex system constituted of several cell types that can be part of the local tissue or recruited during tumor development. Certainly, TME involves multiple components of stroma cells and non-stroma cells [49]. The heterogeneous cellular TME elements mainly contain cancer-associated fibroblasts, endothelial cells, and infiltrating immune cells such as T-cells, B-cells, Dendritic cells (DC), and myeloid cells. These cells, individually or collaboratively, may contribute to generating an immune-suppressive TME that further supports cancer development and progression [50]. Despite cancer cells expressing antigens that can be recognized by the immune system, immunosuppressive TME protects cancer cells from immunosurveillance and prevent the success of immunotherapies [51].

TGF-β has been indicated as a critical player in the generation of an immune-tolerant TME. This factor may either regulate the immune response by acting on the cancer cells, such as downregulating the major histocompatibility complex (MHC) Class I molecules expression, or by influencing the systemic and tumor local response in cancer patients [2, 3, 47, 51, 52]. TGF-β is actively involved in promoting an immunosuppressive TME by controlling the different immune types of cells, including macrophages, DC, and T-cells [53].

TGF-β isoforms have been described as critical regulators of the immune system. The lethal multi-organ inflammation observed due to a massive T-cell dysregulation in the β1-/- mice have pictured TGF-β as a master regulator of mammalian immune system function and homeostasis [54, 55]. Similar inflammatory phenotypes were described later in a smad3 knock-out mouse. The animals died due to multi-organ inflammatory injuries, partly because of severe responsiveness and chemotaxis defects in neutrophils, T-cells, and B-cells [56, 57]. In addition, smad3-/- T-cells exhibit deficiency in their capacity to differentiate towards Tregs, but they were able to acquire a lymphocyte T helper (Th)17 phenotype after TGF-β and interleukin (IL)-6 treatment [5]. Furthermore, this cytokine regulates the functionality of a broad variety of immune cells, such as T-cells, natural killers (NK), DC, neutrophils, and monocytes/macrophages (Fig. 17.1) [58]. Therefore, TGF-β can regulate the initiation and stimulation of innate and adaptive immune responses impacting the anticancer immune cell activities and finally affecting immunotherapies [3, 59].

17.4.1 TGF-β Regulates T-cell Activities and Function

TGF-β regulates almost all critical steps of T-cell anticancer events. Thus, this factor impacts T-cell activation, proliferation, and differentiation, as well as their migration to tumor mass and tumor-draining lymph nodes [3]. In cytotoxic CD8+ T-cells, one of the primary anticancer cellular effectors, TGF-β represses its cell-mediated antitumor activities through direct interaction or indirectly, controlling the accessory cells that rule CD8+ T-cell activities [3, 60]. The TME-associated TGF-β represses the cytolytic T-cell gene program inhibiting then
perforin, granzyme A and B, interferon-γ, and Fas ligand expression [61]. Moreover, this cytokine blocks IL-2 and IL-2 receptor expression via inhibition of the nuclear translocation of the nuclear factor of activated T-cells (NFAT) nuclear translocation in effector and memory T-cells [62, 63]. In addition, the tumor-specific cytotoxic T-cell proliferation is suppressed by TGF-β due to the inhibition of c-Myc and promotion of Smad3-Foxp1 interaction, which abrogated the tumor-reactive T-cells proliferation in vivo [64]. Besides, SH2 domain-containing protein tyrosine phosphatase-1 is induced by TGF-β and recruited to the immunological synapse. This protein binds Tyr phosphorylated domains in the chains of the T-cell receptor (TCR) complex and dephosphorylates them to inhibit the response to external signals [65–67]. Therefore, increased TME TGF-β levels impede the capacity of T-cells to lead an anti-cancer cytolytic response and promote cytotoxic T-cell exhaustion [60].

17.4.2 TGF-β Promotes the Generation of CD4 + Regulatory T-cells

Treg frequency is increased in cancer and participates in the production of tumor antigens tolerance microenvironment, facilitating the escape of cancer cells from immunosurveillance [2]. Given Tregs are generated from a specialized cluster of differentiation (CD)4+ T-cells subset, TGF-β impedes uncommitted CD4+ T-cells from adopting cytotoxic-T-cell-like and NK-like phenotypes, which are involved in tumor eradication [3, 68, 69]. The generation of the CD4+ CD25+ Tregs is mediated by TGF-β lies in Smad3 interaction and activation of the Treg signature transcription factor Foxp3 promoter [70, 71]. Furthermore, TGF-β promotes Treg differentiation over Th17 T-cells generation by inhibiting IL-23R expression and increasing Foxp3 expression that antagonizes the transcription factor RORγt expression [72]. Interestingly, thymus-derived Tregs and induced Tregs may suppress immune responses via the production and activation of TGF-β suggesting positive feedback between TME-associated TGF-β and Treg expression [70].

17.4.3 TGF-β Regulates Natural Killer Cell Function and Activity

Natural Killers are a group of innate immune cells with cytolytic and cytokine-producing capacities against virus-infected cells and cancer cells [73]. TGF-β can exert its immunosuppressive action on NK cells by influencing function metabolism [74]. The ex vivo treatment of NK with TGF-β reduced their antitumoral capacities by decreasing NK group 2 member D (NKG2D) and Nkp30 receptor expression, both membrane receptors that recognize stressed and tumor cells [75]. Consistently, TGF-β has been implicated in the downregulation of NK-associated NKG2D expression in a mouse model of head and neck squamous cell cancer. At the same time, a high TGF-β level of peripheral
circulation of human head and neck squamous cell cancer patients is related to a reduced NK expression of receptor NKp46, a primary NK cell-activating receptor in fighting tumor cells [60, 76–78]. From the metabolic point of view, TGF-β significantly reduces in IL-2-stimulated NK cells the glycolysis, oxidative phosphorylation, and respiratory chain in a mTORC1-independent manner. These effects involve Smad2,3 effectors paralleled to CD71, interferon (IFN)λ-γ, and granzyme B inhibition [79]. In addition, another investigation has shown the role of mTORC1 signaling in NK cell metabolism under the TGF-β challenge. In a model of metastatic breast cancer, peripheral NK cells exhibited low metabolism and mTORC1 response, which was rescued by TGF-β neutralization, resulting in the recovery of oxidative phosphorylation, mTORC1 activity, and IFN-γ production [80]. These observations are consistent with the fact that, in mice and humans, TGF-β inhibits IL-15-induced mTORC1 activation. Furthermore, NK TβR2 deletion increases NK-associated mTORC1 and cytotoxic activities in response to IL-15, along with metastasis inhibition in vivo [81]. In genetically engineered mouse cancer models, the TGF-β/Smad4 axis induces NK cells to acquire a type 1 innate lymphoid cells phenotype disabling cytotoxic functions to control viral infection and tumor metastasis [82, 83].

17.4.4 TGF-β Represses Dendritic Cells Functionality

Dendritic cells are crucial in immunity and immune surveillance; they recognize foreign antigens or aberrant host antigens. Moreover, as antigen-presenting cells, DC mediate tumor immunity via CD4+ and CD8+ T-cell activation [84 and references therein]. TGF-β represses the expression of MHC class II, CD40, CD80, and CD86, and tumor necrosis factor (TNF)-α, IL-12, and CCL5/Rantes in tumor-associated DCs, thus, impairing their antigen-presenting function. This renders DCs functionally defective due to their immature phenotype, which in turn, results in a more tumor-permissive immune microenvironment [60, 85, 86]. Furthermore, tumor-derived TGF-β inhibits plasmacytoid DC production of inflammatory cytokines, such as IFNα, TNF-α, and IL-6, promoting immune tolerance in ovarian cancer [87]. In murine models of breast cancer and melanoma, the loss of TβR3 and its extracellular domain in cancer cells enhances TGF-β signaling in plasmacytoid DC. This results in increases in IDO and CCL22 chemokine expression in plasmacytoid and myeloid DCs, respectively, which results in the promotion of Tregs tumor infiltration and suppression of antitumor immunity [88]. Remarkably, intratumoral DCs are also a source of TGF-β, enhancing Treg differentiation and potentially inducing MDSC expansion and function [85]. Furthermore, TGF-β switches the DC phenotype towards an immature myeloid cell phenotype via upregulation of the Inhibitor of Differentiation-1, which increases immunosuppression during tumor progression [89].

17.4.5 TGF-β1 Increases the Expansion and Function of Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells are composed of populations of myeloid progenitor cells and immature myeloid cells, mainly characterized by the absence of differentiation markers of mature myeloid cells, demonstrating immunosuppressive activities [90, 91]. TGF-β has been involved in the generation, expansion, and function of MDSC. For instance, TGF-β combined with GM-CSF induces the accumulation of monocytic (M)-MDSC from human monocytes in vitro with an effective suppressive capacity of T-cells proliferation while causing Treg generation [92, 93]. Interestingly, B regulatory cells (Bregs), by producing TGF-β educate and enhance polymorphonuclear (PMN)-MDSC and M-MDSC to inhibit T-cell proliferation and cytotoxic CD8+ T lymphocytes differentiation, indicating a different cell interaction in the creation of an immunosuppressive environment [94]. Furthermore,
MDSC-derived from NSCLC patients expresses and secretes TGF-β, which, in an autocrine manner, promotes CD39+/CD73+ MDSC by the hypoxia-inducible factor (HIF)-1α/mTOR axis along with an increased capacity to inhibit T-cell activation and reduced NK cells activities [95]. Moreover, in melanoma, paracrine M-MDSC-produced TGF-β inhibits T-cell proliferation ex vivo [91, 96, 97]. In addition, an autocrine feedback loop may occur in MDSC generation. The MDSC generated from monocytes after TGF-β treatment shows increased TGFB1 gene transactivation and protein production, which consolidate the immunosuppressive function and phenotype of M-MDSC [93]. In addition, MDSC-secreted TGF-β promotes the expression of programmed cell death protein-1 (PD-1) in CD8+ T-cells, which promotes PD-1/PD-ligand-1 (PDL-1) therapy resistance in the TME supporting the notion of TGF-β as a therapeutic target in cancer immunotherapy [98].

17.4.6 TGF-β Regulates Tumor-Associated Macrophages Polarization and Function

Among tumor-associated immune stroma, tumor-associated macrophages [TAMs] are the primary immune cell components that infiltrate tumors and seem crucial to cancer cell proliferation, progression, and metastasis [51, 99]. TAMs are mobilized from bone marrow to infiltrate tumor stroma in response, in part, to tumor-derived inflammatory CC chemokines. At the same time, their survival depends on TME-associated cytokines such as CSF and VEG [100]. Within the TME, several signals influence a wide array of transcriptional networks that allow macrophages to display polarized phenotypes. Macrophages adopt a classical activated M1, with antitumoral activities, and alternatively activated M2 phenotypes, with demonstrated pro-tumoral functions [101]. Nevertheless, macrophages may exhibit several inter-polarized or subtype phenotypes that need to be investigated [102]. High TAM tumor infiltration usually is associated with poor clinical prognosis in most solid cancers [103]. TME-associated TGF-β promotes monocyte recruitment and induces differentiation towards macrophages [104]. Then, these macrophages differentiate into a TAM2-versus-TAM1 phenotype in response to TGF-β [2]. Also, the specific macrophage TβR2 deletion disables cells to differentiate into TAM1, along with the increase in antigen-antitumorigenic TAM functions [105, 106]. Furthermore, TAM2 cells may produce TGF-β that further exacerbates the immunosuppression and contribute to establishing an immunotolerant TME [2, 107].

17.4.7 Tumor-Associated Neutrophils and TGF-β

Neutrophils account for 50–70% of circulating leukocytes, have strongly associated with cancer cells and tumor vasculature, and play crucial roles in tumor growth and progression [108]. Similar to macrophages, neutrophils may also adopt an antitumoral phenotype (named N1) and protumoral phenotype (termed N2) [109]. Depleting tumor-associated neutrophils displaying an N2 phenotype (TAN2) reduces tumor growth and immunosuppressive TME and increases cytotoxic T lymphocyte activities [110, 111]. In turn, tumor-associated neutrophils adopting an N1 phenotype (TAN1) exhibit killing tumor cells capabilities associated with direct or antibody-dependent cytotoxicity and promote adaptive immune cell activation, including T-cells, NKs, and DCs [112].

TGF-β has been demonstrated to be one of the central modulators of neutrophil polarization. TGF-β blockage increases TAN1 neutrophil accumulation, which leads to increased cytotoxicity and activation of CD8+ T cells [109, 113]. Further, TGF-β decreases tumor-suppressive neutrophil roles by inhibiting its capacity to eliminate Fas-ligand-expressing cells, which collaborate further to create a permissive and supportive stroma for tumor progression and metastasis [114].
17.5 Perspectives for Targeting TGF-β1 in Cancer Immunotherapies

Due to TGF-β generating a permissive and immunosuppressive TME, it is an attractive target for cancer immunotherapies. For decades, a vast number of laboratories have dedicated their work to unveiling the leading cellular and molecular mechanisms involved in the TGF-β roles in cancer. This endeavor has been crucial to understanding the TGF-β role in the microenvironment and the development of effective therapeutic strategies to inhibit TGF-β signaling and attenuate tumor growth and progression. Relating to all intratumoral cellular components (i.e., cancer, stromal and immune cells).

Several strategies have been developed as anti-TGF-β oncotherapies. These include TGF-β-neutralizing antibodies, peptide inhibitors, TGF-β receptor kinase inhibitors, antisense oligonucleotides, TGF-β ligand traps, and, more recently, bifunctional antibodies/biomolecules and bifunctional chemical inhibitors. These developed compounds may reduce the excessive TGF-β production and bioavailability and interfere with TGF-β signaling pathways in cancer patients [106, 115–119]. All these therapeutic policies have led to a constant increase in clinical studies, either as monotherapies or in combination with different chemotherapies that are reviewed by Teixeira et al. [120], Derynck et al. [3], and Huang et al. [121]. In the last decade, due to the critical role of TGF-β in cancer immunosuppression, high expectations have been set in combining anti-TGF-β approaches with current immunotherapies strategies.

17.5.1 Immune Checkpoint Immunotherapy and TGF-β

Immunotherapy strategies have provided new insights into the fight against cancer. The discovery of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and PD-1 immune checkpoints [122] propelled the development of immune checkpoint inhibitors (ICIs) [123], which specifically block proteins that disable the immune system to attack cancer cells, has become a breakthrough in tumor therapy [124]. Currently, several ICIs have been developed and are in use in clinical trials. Indeed, in 2011 the first antibody to sequester and block CTLA-4, ipilimumab, thus hindering its binding to cell surface B7 protein, was authorized and followed by the generation of antibodies against PD-1 and PDL-1, pembrolizumab and nivolumab, and atezolizumab and durvalumab respectively, increasing then the immunotherapy armament [125, 126].

The immune system has a mission to recognize and eliminate non-self-immunogenic elements to preserve healthy homeostasis. Primarily, tumor-associated antigens are expected to trigger an anti-tumor response in a cancer-immunity cycle model [127, 128]. This model considers that cancer-associated antigens or neo-antigens are recognized, seized, and processed by DC. Next, DCs present these processed cancer antigens to the MHC proteins to the peripheral lymphoid organs’ naïve T-cells. These cells prime and activate T-cells to specifically recognize cancer-associated antigens and infiltrate tumor mass to exert cytotoxic activities on the cancer cells. Also, more tumor antigens are released and increasing the magnitude of the anti-tumor immune response [127, 128]. However, the upregulation of immune checkpoints prevents effector T-cells to trigger immune cytotoxicity responses to fight cancer. Interestingly, these co-inhibitory signals usually counterbalance co-stimulatory signaling to preserve peripheral tolerance and avoid anti-immune diseases [129]. Thus, cancer cells expressing immune checkpoints result in immune surveillance escape and favor tumor progression and metastasis [128].

The critical role of TGF-β in cancer immunosuppression makes it a promising combining target with ICIs immunotherapy strategies to prolong anticancer immune responses (Fig. 17.2) [120]. In this sense, increased cancer-associated TGF-β signaling contributes to the intrinsic
resistance to ICIs [3]. At the same time, anti-PD-1 antibody therapy is improved by combination with anti-TGF-β neutralizing antibodies in xenograft models of skin cancer [130]. A clinical trial [NCT02947165] that combines anti-PD-1 (spartalizumab), and anti-TGFβ1/2 antibodies (NIS793) has been initiated in patients with advanced malignancies. Advanced therapeutic strategies consider bifunctional fusion proteins composed of TβR2 ligand-binding domain, which function as a TGF-β “trap,” or anti-TGF-β antibodies combined with immune checkpoints-blocking antibodies, have also been effective in both preclinical models and clinical trials [60]. For instance, anti-CTLA4-TβR2 bifunctional fusion protein inhibits Tregs and Th17 cell differentiation. This increases tumor-specific IFNγ+ effector and memory cells, improving the anti-tumor response in melanoma and triple-negative melanoma breast cancer lines resistant to immune checkpoint blockade [131]. Moreover, the first-in-class PD-L1/extracellular domains of human TβR2 bifunctional fusion protein M7824 (MSB0011359C) improve the immuno-therapy response in murine breast and colon cancer models with increased intratumoral T-cell and NK cell activation [132]. Furthermore, in 2015, a phase I clinical trial was launched to treat patients with metastatic or locally advanced solid tumors with M7824 [NCT02517398]. The first data indicate that M7824 appears to have a manageable safety profile in cancer patients. This bifunctional fusion protein can saturate PD-L1 in peripheral blood mononuclear cells and clear all three plasmatic TGF-βs [133].

17.5.2 Adoptive Cell Therapy/Chimeric Antigen Receptor (CAR) T-Cell Therapy and TGF-β

A new revolutionary therapeutic technique has recently emerged, the chimeric antigen receptor (CAR) T-cells, which provides further insight into adoptive cell therapy (ACT) for cancer [124]. From a simple view, the CAR construct results from the fusion of a specific anti-tumor-associated antigen (TAA) antibody polypeptide chain with the TCR/CD3ζ signal-mediated activating machinery of the T cell. Also, it may contain one or more domains derived from co-stimulatory T cell receptors CD28, 4-1BB, or OX40. Then, the TAA-CAR-specific construct is ectopically expressed in the immune effector cells to recognize and potentially target cancer cells. When CAR T cells bind to TAA at the cancer cell surface, they proliferate and kill tumor cells. CAR T cells represent a significant advancement in cancer immunotherapy and a genetic engineering platform to develop CAR-based immunotherapies using other immune cells [134].

On the other hand, immune suppression mediated by TGF-β is one of the main obstacles to the success of adoptive cell therapy for the treatment of solid tumors. Adoptive transfer that considers ex vivo expanded genetically engineered T cells with chimeric antigen receptors combined with TGF-β inhibition is now considered for cancer immunotherapy [135] (Fig. 17.3). Recently, Golumba-Nagy et al. [135] have developed CAR T-cells with engineered modification of CD28 by site-directed mutagenesis (CD28-ζ CAR) that avoids TGF-β-mediated repression and may improve CAR T-cell activity against tumors with elevated TGF-β expression. In addition, CRISPR/Cas9-mediated knock out of TβR2 in CAR T cells, reduces the induced Tregs conversion and prevents CAR T-cell exhaustion. In vivo, TβR2-edited CAR T-cells show improved capacities for tumor elimination of either cell line-derived xenograft or patient-derived xenograft solid tumor models. When a PD-1 deletion was engineered to generate PDCD1/TGFBR2 double-KO CAR T-cells, CAR T-cell resistance to suppressive TME improved dramatically [136].

A dominant-negative form of TβR2 co-expressed along with CAR in T-cells directed to prostate-specific membrane antigen (CART-PSMA-TGFβRDNC cells) has shown increased proliferation, exhaustion resistance, and enhanced cytokine release by engineered lymphocytes. These characteristics accompanied
long-term in vivo survival and tumor abolition in aggressive human prostate cancer mouse models. Due to the great expectation created by the potency of preclinical results, a phase I clinical trial was initiated in 2017 to treat patients with relapsed and refractory metastatic prostate cancer (NCT03089203) [50]. Moreover, CAR T-cells that express an extracellular single-chain variable fragment based on the sequence of three TGF-β-blocking antibodies that neutralize the active form of human TGF-β (TGF-β CAR) have been produced. These molecules inhibit endogenous TGF-β signaling and stimulate the engineered TGF-β CAR T-cells through CD28-signaling, which reconverts TGF-β binding from inhibitory to T-cell activating signaling. In this case, TGF-β potently stimulates T-cells to proliferate and secrete Th1 cytokines. Thus, TGF-β CAR-T cells exert an immunosuppressive role in the presence of TGF-β1 [137]. Furthermore, TGF-β CAR-T cells protect tumor-associated immune cells from TGF-1 suppressive effects since in the presence of TGF-β, tumor-targeted CD8+ T-cells retain cytolytic activity and disable CD4+ T-cells differentiation towards Tregs [137]. The inhibition of TGF-β signaling by the TβR1 inhibitor galunisertib enhances CD133 and Her2 CAR T-cells anti-cancer activity in glioma and breast cancer cell lines [138]. Therefore, this may establish new routes and encourage clinical use of engineered CAR T-cells with anti-TGF-β function to target immunosuppressive TME resulting from increased levels of TGF-β.

17.6 Concluding Remarks

During the last decades, fruitful efforts have been made to unveil the underlying cellular and molecular implicated in the main aspects of the TME-associated immunosuppressive environment. In this sense, this review described and
discussed the aspects involved in the diverse roles of TGF-β in the generation of immune permissive TME, in part due to immunosurveillance repression, which favors cancer cells to escape from immune attack and enables tumor progression and metastasis. TGF-β seems to participate in regulating cellular and non-cellular compartments in the tumor mass. Beyond cancer cells and extracellular matrix regulation, TGF-β strongly influences the function and activities of almost all immune cells infiltrating within the tumor stroma, such as inhibiting T-cells, NKs, and DCs activation and function, while promoting the generation of immune cells with immunosuppressive activities, including Tregs, TAM1 and TAN1 cells, which creates immune-tolerant conditions protecting cancer cells from immune clearance.

All these immunoregulatory activities of TGF-β make it an attractive target for improving current immunotherapies. Many clinical trials have been implemented as combined strategies considering TGF-β inhibition and ICIs immunotherapy. Also, combined preclinical
studies have combined TGF-β inhibition with the CAR-T approach. In both cases, combining TGF-β targeting with ICIs or adoptive cell therapy may negatively impact the amplification loop between TGF-β and cancer cells, inhibiting T-cell activation and cytotoxicity functions. Therefore, these combined therapies may limit tumor progression and metastasis, impairing tumor immunosurveillance escape and body dissemination.

Although CAR-T has shown to be promising in treating hematological malignancies, this strategy tackled some challenges in being implemented in solid tumors [139]. Solid tumors comprise heterogeneous cell populations, a complex, dense extracellular matrix, a hypoxic microenvironment, and an elevated interstitial pressure, which prevent T-cell recruitment, activation, and cytotoxic persistence while simultaneously promoting the recruitment of immune suppressor cells [140]. These aspects also dampen CAR T cell antitumor activities, and new adoptive cell therapies have been developed. For instance, besides displaying professional antigen-presenting cell [APC] activities, macrophages exhibit a high capacity to migrate and infiltrate the tumor mass. Recently the concept of CAR-M has been introduced [141, 142]. Moreover, this technology can include other immune cells, such as natural killer (NK)/NKT cells, γδ T cells, DCs, macrophages, and Tregs [143]. Nevertheless, all these cells are under the influence of TGF-β, indicating that manipulating TGF-β function and activity may be a therapeutical requirement to improve the success of cancer immunotherapies.

Finally, clarifying the complex immunoregulatory network of TGF-β in tumors is critical for understanding the contribution to cancer initiation, progression, immune resistance, and metastasis. This may reveal new potential clinical treatments to increase the success of current chemotherapies and immunotherapies in fighting cancer growth and metastasis.

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