Sphingosine-1-Phosphate and Its Receptors: A Mutual Link between Blood Coagulation and Inflammation

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Sphingosine-1-phosphate (S1P) is a versatile lipid signaling molecule and key regulator in vascular inflammation. S1P is secreted by platelets, monocytes, and vascular endothelial and smooth muscle cells. It binds specifically to a family of G-protein-coupled receptors, S1P receptors 1 to 5, resulting in downstream signaling and numerous cellular effects. S1P modulates cell proliferation and migration, and mediates proinflammatory responses and apoptosis. In the vascular barrier, S1P regulates permeability and endothelial reactions and recruitment of monocytes and may modulate atherosclerosis. Only recently has S1P emerged as a critical mediator which directly links the coagulation factor system to vascular inflammation. The multifunctional proteases thrombin and FXa regulate local S1P availability and interact with S1P signaling at multiple levels in various vascular cell types. Differential expression patterns and intracellular signaling pathways of each receptor enable S1P to exert its widespread functions. Although a vast amount of information is available about the functions of S1P and its receptors in the regulation of physiological and pathophysiological conditions, S1P-mediated mechanisms in the vasculature remain to be elucidated. This review summarizes recent findings regarding the role of S1P and its receptors in vascular wall and blood cells, which link the coagulation system to inflammatory responses in the vasculature.

1. Introduction

Sphingosine-1-phosphate (SIP), a highly active lipid mediator, exhibits a broad range of cellular activities including proliferation, survival, adhesion, and migration [1, 2]. SIP is critical for mammalian cardiac development and for maturation of the systemic circulatory system [3]. These biological actions are carried out by predominantly intracellularly produced SIP via sphingosine kinase (SphK), of which two isoforms SphK1 and SphK2 exist [4, 5]. Moreover, SIP has emerged as an intracellular second messenger involved in regulation of cell proliferation and in mobilization of internal calcium stores by a protein kinase C independent pathway [6]. Further reports suggest that SIP found within the extracellular space is not merely derived from intracellular generation but biosynthetic enzymes of the SIP metabolism appear to subsist in the extracellular space [7]. Indeed, the majority of studies have focused on the functions of extracellular SIP. This “outside the cell” SIP acts in an autocrine or paracrine manner as an agonist for a unique family of G-protein-coupled receptors which to date comprises the five SIP receptors (SIPRs) SIPRI–SIPR5 [8, 9]. Extracellular SIP regulates proliferation and migration of vascular endothelial cells (ECs) [10] and smooth muscle cells (VSMCs) [11] and critically determines lymphocyte egress and angiogenesis [12]. Both SIP and SIPRs regulate vascular tone either by directly modulating the smooth muscle layer or by stimulating ECs to release bioactive molecules which regulate VSMCs responses in a paracrine manner [13].

The levels of SIP in plasma and tissues are tightly regulated by the balance between its synthesis by sphingosine
kinases and degradation [2, 14]. The role of S1P and the processes involved in its biosynthesis, that is, regulation of the metabolizing enzymes, for controlling vascular integrity has been studied thoroughly in vitro and in vivo [15]. Vascular proliferative disorders such as atherosclerosis and persistent proinflammatory challenges of the vessel wall [16] are characterized by the activation of the coagulation cascade and platelet activation, both processes which elevate local S1P concentrations [17]. This may play an important role in directing immune cells to sites of local injury and directly links the coagulation system to S1P-mediated inflammatory responses in vivo. After vascular injury, the coagulation cascade is initiated by activating the clotting factors X (FXa) and ultimately thrombin, which are both key regulators of subsequent tissue repair and remodeling [18, 19]. FXa-mediated thrombin generation initiates and is itself amplified by subsequent platelet activation, finally leading to cleavage of fibrinogen and eventually the formation of the mural thrombus [20, 21]. In addition to their physiological function in hemostasis, the clotting proteases thrombin and FXa are also accountable for clinically relevant pathological responses such as postphlebitic inflammatory and tissue repair reactions [16, 22]. The biological effects of FXa and thrombin are mediated via a family of G-protein-coupled receptors, protease-activated receptors 1, 2, 3, and 4 (PAR-1–PAR-4) [23, 24]. Thrombin initiates signaling through PAR-1, PAR-3, and PAR-4, while FXa acts via PAR-1 and PAR-2. Previous reports, including studies from our group, have reported that PARs stimulate VSMCs proliferation and migration, modify the composition of the extracellular matrix of blood vessels, and mediate proinflammatory responses in the vessel wall [25–28]. Because proliferation and migration of VSMCs are considered key events in the development of atherosclerosis and vascular remodeling, these cellular effects of thrombin and FXa may directly contribute to the pathogenesis of vascular diseases such as progression of atherosclerosis and restenosis after vascular injury. In addition, recent studies highlight numerous interactions between blood coagulation and the S1P signaling system [17, 29].

This review discusses the recent findings concerning the role of S1P and its receptors in vascular and blood cells which are interlinked with the coagulation system. Particularly, hemostasis-related mechanisms which increase local S1P availability and the regulation of PAR receptor expression by S1P are highlighted. Elucidating the complex interactions between blood coagulation and the S1P signaling network further may bear the potential to discover and develop novel targets for the therapy of inflammation-prone vascular diseases.

2. Biosynthesis, Degradation, and Functions of S1P in the Vascular System

S1P biosynthesis is tightly interlinked with the metabolism of ceramide. Ceramide is formed either de novo from serine, palmitoyl CoA, and fatty acid or from breakdown of membrane-resident sphingomyelin [30, 31]. Ceramide is further converted to sphingosine by enzymatic action of ceramidase. Finally, the bioactive lysophospholipid S1P is produced by phosphorylation of sphingosine. This reaction is catalyzed by the two sphingosine kinase isoenzymes SphK1 and SphK2. Maintaining a balance between S1P generation and degradation is critical for regulation of cell growth and plays a key role in pathological processes such as carcinogenesis [32]. S1P degradation is achieved via reversible dephosphorylation by two S1P-specific phosphatases (SPP1 and SPP2) or irreversible hydrolysis by S1P lyase. S1P exerts actions either by binding to its intracellular targets or through its specific receptor in autocrine, paracrine, and/or endocrine manner [31].

S1P is secreted, stored, and exported by the cells of the vessel wall, VSMCs, and ECs, respectively. Recent observations highlight the critical role of the putative S1P transporter spinster homolog 2 (Sphn2) in endothelial S1P release and in lymphocyte trafficking [33, 34]. In other cell types, that is, breast cancer and mast cell, the ABC (ATP-binding cassette) transporter family members ABCCl and ABCG2, known regulators of inflammatory processes, facilitate export of S1P across the cell membrane [35, 36]. S1P regulates a diverse range of cellular processes that are important in immunity, inflammation, and inflammatory disorders [37, 38]. Once secreted, most of the S1P binds to albumin or serum lipoproteins [39]. Whether this carrier-bound serum S1P or rather locally produced S1P is important for the diverse cellular functions is a matter of current debate [40]. The metabolism and distinct vascular functions of S1P are highlighted in Figure 1.

3. Interactions of S1P Receptors and Thrombin Receptors Affect Endothelial Function

Endothelial cells synthesize and secrete large amounts of S1P and contribute substantially to generating the high S1P level present in the blood [40, 41]. Of the five S1PRs, endothelial cells express S1PR1, S1PR2, and S1PR3 [29]. S1P modulates diverse endothelial activities including proliferation [42], survival [43], migration [44], and regulation of proinflammatory responses [45] and controls the endothelial barrier function [46–49]. S1PR1 is highly expressed in endothelial cells [50] and regulates cytoskeletal structure, migration, and vessel maturation [51, 52]. In S1PR1 receptor deficient embryos, blood vessels were incompletely covered by VSMCs, indicating that S1PR1 also regulates vascular maturation [53]. Thus, S1PR1 appears to mediate predominantly physiological functions while particularly S1PR2 regulates inflammatory endothelial responses and is upregulated during inflammatory conditions such as atherosclerosis [45, 54]. These assumptions are in agreement with recent observations of varying S1P concentrations resulting in differential receptor activation [55] and the differential regulation of S1PR1 and S1PR2 expression during conditions of hyperglycemia-induced endothelial cell dysfunction [56].

A key regulator of endothelial function is the coagulation system with factors such as thrombin known to affect its permeability [57] as well as endothelial inflammation [58]. Thrombin causes induction of endothelial cell contraction
Figure 1: Synthesis of S1P and functions in the vascular system. De novo ceramide synthesis in general originates from condensation of serine, palmitoyl CoA, and fatty acid, a multistep enzyme catalysed process. Ceramide can be converted reversibly into sphingomyelin by sphingomyelinase or to glycosphingolipids. It is further metabolised by ceramidase to sphingosine, which can then be phosphorylated into S1P by sphingosine kinase isoforms 1 and 2 (SphK1/2). This phosphorylation can be reverted by the S1P phosphatases SPP1 and SPP2 or irreversible degradation by S1P lyase can occur. S1P may act intracellularly or is exported out of the cell via ABC transporters or Spns2, dependent on the cell type, and may bind to one of its receptors (S1PR1–S1PR5) to initiate G-protein mediated signaling. S1P modulates key processes of vascular pathogenesis which involve but are not restricted to modulation of cell proliferation and migration and regulation of vascular tone and immune functions.

Figure 2: Thrombin effects in endothelial cells involve S1P signaling. Activation of the classical thrombin receptor PAR-1 interrupts endothelial barrier integrity by induction of endothelial contraction through stimulation of G12/13 subunit and Rho signalling pathway (left). Conversely, thrombin also induces expression of SphK1 and increases S1P generation. This involves PAR-1-induced signaling via activated protein C (APC) and the endothelial protein C receptor (EPCR). S1P in turn transactivates S1PR1 leading to Gi-dependent activation of the Rac-1 signaling pathway. This effect improves endothelial integrity to counteract and limit thrombin-induced endothelial damage and vascular leakage (right).

and disruption of endothelial barrier integrity via the PAR-1 receptor [57]. This involves signaling through the endothelial protein C receptor and includes cross talk with the SIPR system [59]. SIP/SIPR signaling can counteract this detrimental effect of thrombin and appears to protect from vascular leakage and tissue damage such as edema formation [60]. Thus, thrombin may enhance endothelial S1P generation and signaling within the endothelium to limit its own actions of inducing vascular leakage via mutual PAR-1 mediated SIP/SIPR1 actions (Figure 2).
of VSMCs functions and vascular development as well as S1P has rapidly been gaining attention as a key regulator of DNA synthesis and migration in VSMCs [66]. Since then, migration. Early studies also suggested a function of S1P for formation [65]. Numerous growth factors and inflammatory mediators of inflammation [64] as well as during vascular lesion formation [63]. The relevance of S1P in the regulation of vascular permeability, lymphocyte trafficking, and vascular development is well documented in vivo [41]. S1PR1 deficiency resulted in impaired vascular maturation [74] whereas SphK1 and SphK2 null mice have shown disturbed angiogenesis resulting in embryonic lethality [75]. Furthermore, Kono et al. reported that S1PR1, S1PR2, and S1PR3 function coordinately during embryonic angiogenesis [76]. Taken together, these studies suggest S1P governs physiological vascular homeostasis and is also an important mediator during pathophysiologic conditions such as inflammation.

The coagulation system has been well recognized as a key regulator of inflammation. An interaction between thrombin-induced PAR-1 signaling and the SIP system via enhanced expression of SphK1 and elevated SIP synthesis has first been observed in epithelial and in endothelial cells [77]. In addition, the SIP system has been suggested as a downstream component of thrombin signaling also in other cell types. Work from Niessen et al. revealed a critical role of cross talk between PAR-1 and the SIP3 receptor in dendritic cells in the amplification of inflammation during sepsis [78]. Further studies indicate direct involvement of thrombin in regulating key processes of cellular proinflammatory responses.

### Table 1: Mechanistic studies which directly link SIP and its receptors to the thrombin or FXa receptors PAR-1 to PAR-4, their (patho)physiological actions, and associated signaling pathways.

| Receptor(s)/stimuli | G-protein binding | Signaling pathway | Physiological action(s) | Reference |
|---------------------|-------------------|-------------------|------------------------|-----------|
| SIP/thrombin        | Not described     | NF-κB, EGR-1/ERK1/2 | Enhanced tissue factor expression in endothelial cells | [59] |
| SIP/thrombin        | GIT1 and GIT2     | Focal adhesion kinase (FAK)/Src | Regulation of endothelial barrier function | [46] |
| SIPR3/PAR-1 (via SphKI) | G12/13 | IL-1B | Induces tissue factor production, inflammation, and coagulation | [37] |
| SIPRI and SIPR3/PAR-1 | G12/13 | Rac-1/Rho | Inflammatory responses | [76] |
| SIP/PAR-1 (via SphKI) | G12/13 | NF-κB | Regulation of endothelial function | [75] |
| SphKI/FXa via PAR-1 and PAR-2 | Gq | Rho-kinase, PKC | Mitogenesis and migration of VSMCs | [79] |
| SIP/FXa             | Gα; G12/13       | Rho-A/GTPases | Proliferation/survival | [80] |
| SIPR3/PAR-4         | G11 | Akt, p38 MAPK | Migration, chemotaxis | [107] |

In certain systemic diseases such as sepsis, signaling through PAR-1 exerts multiple and partly opposing functions. This has been attributed to either promoting dendritic cell-dependent coagulation and inflammation or reducing sepsis lethality due to protein C activation and involves regulation of the balance between differential vascular SIPR (SIPRI/SIPR3) signaling pathways [59]. Thus, not only PAR signaling but also SIP actions in endothelial cells appear to involve opposing mechanisms and cellular effects. On the one hand, SIP enhances barrier integrity to counteract thrombin-mediated disturbance of permeability to restore vascular homeostasis after injury; on the other hand, it synergizes with thrombin in upregulating the expression of TF in endothelial cells [61]. Thereby, SIP may enhance generation of thrombin under proinflammatory conditions such as atherosclerosis. In this context, a recent study from Campos et al. is of interest, which showed that the functional SIP receptor antagonist fingolimod [62] reduces infarct size and enhances blood-brain barrier integrity in rodent models of stroke [63]. To determine whether this observation, besides an effect on barrier function, involves direct thrombotic or antithrombotic mechanisms of SIP signaling requires further investigations. The mechanistic studies which directly link SIP and its receptors to the thrombin or FXa receptors, their (patho)physiological actions, and associated signaling pathways are summarized in Table 1.

**4. Role of Coagulation Factor-SIP Interactions in the Vessel Wall**

Proliferation and migration of vascular VSMCs are fundamental features in physiological processes such as maturation of blood vessel [64] as well as during vascular lesion formation [65]. Numerous growth factors and inflammatory molecules like cytokines regulate VSMCs proliferation and migration. Early studies also suggested a function of SIP for DNA synthesis and migration in VSMCs [66]. Since then, SIP has rapidly been gaining attention as a key regulator of VSMCs functions and vascular development as well as a critical factor for vascular damage. Like endothelial cells, VSMCs obtained from different vascular beds express SIPRI, SIPR2, and SIPR3 receptors [67–71]. Kluk and Hla reported that SIP via activation of SIPRI significantly stimulates both proliferative and migratory responses for VSMCs [70]. This is in agreement with the observation that SIP induces VSMCs migration through a Gαi-linked, Ras- and PI3-K-coupled, ERK1/2-dependent process [71]. A further role of SIPR2 receptor in vascular physiology and pathology has been established through regulation of intracellular signaling pathways, such as Rho GTPase, the phosphatase PTEN, and VE-cadherin pathways [72]. Nodai et al. found high mRNA levels of the receptors SIPR2 and SIPR3 in rat VSMCs [73]. They suggest that predominantly SIPR3 stimulates expression of COX-2 through mechanisms involving calcium-dependent PKC and Src-family tyrosine kinase [73].
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cells, wasseenatFXaconcentrations (3to30nM) whichhave
(Figure3) [81]. This stimulatory effect, observed in cultured
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possibly modification of S1P-mediated signaling in patients
calculating inVSMCs. During vascular injury, local generation of

in VSMCs. This involves activation of classical inflammatory
transcription factors such as NF-κB [79], but also immune
regulators that have more recently become of interest, that is,
the forkhead-box-O transcription factor family [80].

In addition to thrombin, FXa can independently activate
PAR-1 and PAR-2. Recent work from our laboratory has
shown that FXa regulates transcription of SphK1 and elevates
SIP biosynthesis in human vascular smooth muscle cells
(Figure 3) [81]. This stimulatory effect, observed in cultured
cells, was seen at FXa concentrations (3 to 30 nM) which have
been shown to occur during thrombus formation ex vivo [26].
Expression of SphK1 by FXa was attenuated by inhibitors of
the Rho-associated kinase and of classical PKC isoforms. In
addition, FXa caused activation of the small GTPase RhoA in
human smooth muscle cells. This is particularly interesting,
because small GTPases are known to play key roles in mediat-
ing signaling responses of the SIP receptor [82], suggesting
a mutual interaction of SIP receptor-initiated signaling and
regulation of SIP synthesis. Interestingly, FX/FXa appears
to be already present within human carotid artery plaques
(plaque material is well known to be highly thrombotic)
and colocalizes with SphK1 expression [81]. The presence
of active coagulation factors in atherosclerotic tissue has
also been shown by others [83]. This observation suggests a
close relation between coagulation factor signaling and pro-
gression of the atherothrombotic disease. Whether possible
antiproliferative or antiangiogenic actions of the novel oral
coaulation inhibitors involve affecting SphK1 expression and
possibly modification of SIP-mediated signaling in patients
requires further investigations.

5. Release Mechanisms of SIP from
Activated Platelets

The biological effects of SIP released from activated
platelets in the vasculature include inhibition of platelet
aggregation [84], angiogenesis, vascular development, and
thrombosis-related vascular diseases such as the acute
coronary syndrome [47, 85, 86]. Platelets were originally
suggested to be the prime source of plasma S1P, because they
exhibit high SphK activity. In human platelets, SphK2 is the
predominant isoform [87]. Surprisingly, however, although
platelets do express SIP receptors [88] during in vitro platelet
function testing such as light transmission aggreometry,
SIP does not appear to function as a potent direct platelet
agonist [89].

Due to lack of SIP lyase activity in platelets [90], SIP
abundantly accumulates intracellularly. However, SIP plasma
levels in thrombocytopenic mice were found to remain
largely unchanged [91], suggesting that resting platelets may
not substantially contribute to circulating SIP concentrations
in plasma. Platelets release huge amounts of SIP during
blood clotting or upon direct activation with agonists of PKC
signaling like thrombin [92, 93]. Work from our laboratory
suggests a critical role of thromboxane in regulating the
release of SIP from human platelets [89]. Secretion of SIP
was induced after activation of platelets with potent agonists
such as thrombin or selective PAR-activating peptides (PAR-
APs) or with a high concentration of collagen. This effect was
largely prevented after inhibition of thromboxane formation
by classical inhibitors of cyclooxygenase-1 (COX-1), such as
aspirin, diclofenac, or ibuprofen (Figure 4 and [89]). Thus,
one pathway mediating release of platelet-derived SIP after
platelet activation depends on COX-1-derived thromboxane.

Since SIP represents an amphiphilic anion, its transloca-
tion across the plasma membrane supposedly does require
active transport proteins. As mentioned above, several studies
in various cell types point to the involvement of a transporter
of the ABC family [94, 95]. However, the biological functions
of these proteins are by far not completely understood.
In activated platelets, SIP secretion was affected by several
compounds that are known to inhibit members of the mul-
tidrug resistance protein (MRP/ABCC) subfamily of ABC
transporters [89]. A variety of transporters including MRPs
are expressed in platelets that exert important functions for
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6. Interactions of the SIP-PAR System for
Inflammatory Monocyte Responses

During vascular inflammation, monocytes secrete sev-
eral proinflammatory cytokines and adhesion molecules,
a response mechanism which facilitates recruitment and
adherence to the inflamed and activated endothelium [99].
Thrombin is one of the key factors controlling the migrat-
ory and secretory behavior of monocytes [100]. Human
peripheral blood monocytes predominantly express PAR-
1 and PAR-3 [101]. Interestingly, during differentiation
into macrophages, that is, by colony-stimulating factors,
the expression levels of PAR-1, PAR-2, and PAR-3 are
highly elevated indicating dynamic adaptation mechanisms
of the system [101]. A recent report indicates that monocytes from patients with antiphospholipid syndrome expressed PAR-1 to PAR-3 but not PAR-4 [102]. Other authors have described a role for PAR-4 in the release of inflammatory markers from monocytes, such as IL-6 [103]. Thus, different PARs may be differentially regulated in response to various stimuli during vascular pathogenesis.

SIP is a recently recognized novel regulator also of monocyte functions [104, 105]. Human monocytes express all five S1PRs at the mRNA and protein levels [106], possibly mediating the regulation of monocyte apoptosis and chemotaxis [107]. In leukocytes, SIP contributes to P-selectin-dependent rolling through endothelial S1PR3 [108]. In dendritic and endothelial cells, involvement of SIP in the signaling pathways of the prototypic thrombin receptor PAR-1 has been suggested [80]. However, little information is to date available about a possible cross talk between SIP and PARs in monocytes. Recent data from our laboratory provide evidence that (i) SIP directly enhances expression of the thrombin receptors PAR-1 and PAR-4 in human monocytes and that (ii) this results in enhanced PAR-4-mediated chemotaxis and elevated generation of COX-2 in response to thrombin [109].

SIP induced PAR-1 and PAR-4 mRNA and total protein expression in human monocytes and U937 cells in a concentration- and time-dependent manner, respectively. However, only PAR-4 cell-surface expression was increased significantly by SIP, whereas cell-surface PAR-1 remained unaffected. This response was associated with activation of the Akt, ERKI/2, and p38 pathway and induction of COX-2 but not COX-1. PAR-4-mediated induction of COX-2 was prevented by pharmacological inhibition of the PI3 kinase pathway and incubation of human monocytes with SIP resulted in an enhanced PAR-4-dependent chemotaxis response to thrombin. Thus, SIP enhances monocyte responses to thrombin via upregulation of PAR-4 protein and cell-surface expression, which promotes migration and COX-2 abundance. These studies establish a direct link between SIP receptor activation and regulation of thrombin receptor expression in human monocyte and the subsequent cellular responses to thrombin. This mechanism may facilitate monocyte recruitment to sites of vessel injury and inflammation (Figure 5).

7. Summary and Perspective

Taken together, complex (patho)physiological interactions between blood coagulation factors and SIP and their respective signaling receptors are being increasingly recognized (see Table 1). This involves regulation of endothelial, smooth muscle, and immune cell functions. Of particular interest for the clinic is the use of new selective modulators of the SIP-S1PR signaling system such as fingolimod as therapeutic agents. In the cardiovascular system, the role of SIP as therapeutic target or as a potential biomarker in cardiovascular diseases is still unclear. For example, the role of SIP levels and release, that is, from thrombin-activated platelets during myocardial infarction, is not finally defined to date. Recent studies indicate that SIP levels substantially vary during cardiovascular disease entities [110, 111]. An important future issue is the definition of circulating SIP levels in defined study populations as well as in clinical cohorts such as patients...
with acute coronary syndrome. The clinical relevance and therapeutic potential of altering SIP levels or receptor activity in atherothrombosis associated diseases is to date unclear and warrants future studies.

**Abbreviations**

ABC: Adenosine triphosphate-binding cassette transporter  
APC: Activated protein C  
COX-I: Cyclooxygenase-1  
EGR-I: Early growth response protein 1  
EPCR: Endothelial protein C receptor  
FAK: Focal adhesion kinase  
FXa: Activated coagulation factor X  
NF-κB: Nuclear factor-κB  
PAR-1/PAR-2/PAR-4: Protease-activated receptor 1/2/4  
p38 MAPK: p38 mitogen-activated protein kinases  
PI3K: Phosphatidylinositol-3-kinases  
PAR-4AP: PAR-4 activating peptide  
PGE2: Prostaglandin E2  
PKC: Protein kinase C  
Rac-1: Ras-related C3 botulinum toxin substrate 1  
Rho: Ras-homologous GTPase family member  
SIP: Sphingosine-1-phosphate  
SIP1–SIP3: SIP receptors 1 to 3  
Sph: Sphingosine  
SphK-I: Sphingosine kinase-1  
Spns2: Spinster homolog 2  
TXA2: Thromboxane A2  
W146: SIPRI1 antagonist  
CAY: SIP3 antagonist  
LY: An inhibitor of PI3K upstream of Akt  
SB: p38 MAPK inhibitor.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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