Targeting RGD-binding integrins as an integrative therapy for diabetic retinopathy and neovascular age-related macular degeneration

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A R T I C L E   I N F O

Keywords:
- RGD-binding integrin
- Diabetic retinopathy
- Neovascular age-related macular degeneration
- Retina

A B S T R A C T

Integrins are a class of transmembrane receptors that are involved in a wide range of biological functions. Dysregulation of integrins has been implicated in many pathological processes and consequently, they are attractive therapeutic targets. In the ophthalmology arena, there is extensive evidence suggesting that integrins play an important role in diabetic retinopathy (DR), age-related macular degeneration (AMD), glaucoma, dry eye disease and retinal vein occlusion. For example, there is extensive evidence that arginyl-glycyl-aspartic acid (Arg-Gly-Asp; RGD)-binding integrins are involved in key disease hallmarks of DR and neovascular AMD (nvAMD), specifically inflammation, vascular leakage, angiogenesis and fibrosis. Based on such evidence, drugs that engage integrin-linked pathways have received attention for their potential to block all these vision-threatening pathways.

This review focuses on the pathophysiological role that RGD-binding integrins can have in complex multifactorial retinal disorders like DR, diabetic macular edema (DME) and nvAMD, which are leading causes of blindness in developed countries. Special emphasis will be given on how RGD-binding integrins can modulate the intricate molecular pathways and regulate the underlying pathological mechanisms. For instance, the interaction between integrins and key molecular players such as growth factors, cytokines and enzymes will be summarized. In addition, recent clinical advances linked to targeting RGD-binding integrins in the context of DME and nvAMD will be discussed alongside future potential for limiting progression of these diseases.

1. Introduction

Integrin receptors are transmembrane heterodimeric adhesion proteins that play an essential role in integrating the extracellular to the intracellular environment. In the mid-eighties, the first integrin receptor was discovered based on its engagement with specific motifs on extracellular matrix (ECM) protein fibronectin (Pierschbacher and Ruoslahti, 1984; Tamkun et al., 1986). Since then, a vast number of articles on integrins have been published on a regular basis (on average ~2000 new publications per year and ~27,000 to date) and it is established that these receptors are involved in various cellular processes such as adhesion, differentiation, shape, migration, signalling, invasion, proliferation and survival with clear linkage to pathological processes, including inflammation, vascular leakage, neovascularization and fibrosis (Eklund et al., 2017; Fu et al., 2007; Hames et al., 1996; Kanda et al., 2012; Koch and Distler, 2007; Santulli et al., 2008; Umeda et al., 2006; Wilkinson-Berka et al., 2006; Zahn et al., 2009). The multi-faceted role integrins play in cell pathophysiology is still being investigated and deciphering the precise nature of integrin-linked molecular mechanisms in health and disease remains a significant and relevant research challenge.
In the last few years, there has been renewed interest in integrins and especially drugs that target arginyl-glycyl-aspartic acid (Arg-Gly-Asp; RGD)-binding integrins in tissues of the eye. This reinvigoration in the area has been driven, at least in part, by recent preclinical and clinical studies which demonstrated promising results in retinal diseases such as diabetic retinopathy (DR), diabetic macular edema (DME) and neovascular age-related macular degeneration (nvAMD) (Askew et al., 2018; Shaw et al., 2020; Tolentino et al., 2016). The main goal of this review is to highlight preclinical as well as clinical knowledge on the role of mainly RGD-binding integrins in DR, DME, proliferative DR (PDR) and nvAMD. In addition, we will endeavour to provide comprehensive information on the intricate cellular and molecular mechanisms of RGD-binding integrin signalling in the main disease hallmarks of these vision-threatening retinal disorders.

2. Classification and function of integrins

Integrins constitute a family of ubiquitously expressed transmembrane receptors that regulate cell-cell and cell-ECM interactions (Bouvard et al., 2013; LaFoya et al., 2018; Moser et al., 2009; Wu and Reddy, 2012). Integrins are obligate heterodimeric receptors consisting of an α- and a β-subunit. Currently, 18 α-subunits and eight β-subunits are known, and various combinations thereof constitute the family of 24 heterodimeric integrin members (Humphries et al., 2006; Pan et al., 2016). The classification of the integrin receptor family into four different classes is based on their structure similarity and ligand recognition pattern: 1) RGD-binding, 2) collagen-binding, 3) leukocyte-specific and 4) laminin-binding integrin receptors (Fig. 1). The first subgroup recognizes the tripeptide sequence RGD in their natural ligands (e.g. fibronectin) and consists of eight different integrins: \( \alpha_v\beta_1, \alpha_v\beta_3, \alpha_v\beta_5, \alpha_v\beta_6, \alpha_v\beta_8, \alpha_v\beta_9, \alpha_v\beta_10 \) and \( \alpha_v\beta_11 \). However, it is now

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**List of abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| AMD | Age-related macular degeneration |
| Ang | Angiopoietin |
| ANGPTL | Angiopoietin-like protein |
| BBB | Blood-brain barrier |
| BCVA | Best corrected visual acuity |
| bFGF | Basic fibroblast growth factor |
| CAM | Chick chorioallantoic membrane |
| CNV | Choroidal neovascularization |
| CST | Central subfield thickness |
| CTGF | Connective tissue growth factor |
| DME | Diabetic macular edema |
| DR | Diabetic retinopathy |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EMT | Epithelial-to-mesenchymal transition |
| EndoMT | Endothelial-to-mesenchymal transition |
| ERK | Extracellular signal-regulated kinase |
| FAK | Focal adhesion kinase |
| HUVEC | Human umbilical vein endothelial cell |
| IGFBP | Insulin-like growth factor-binding protein |
| IL | Interleukin |
| IPL | Inner plexiform layer |
| IVT | Intravitreal |
| LAP | Latency-associated peptide |
| MCP | Monocyte chemoattractant protein |
| MMP | Matrix metalloproteinase |
| nAMD | Neovascular age-related macular degeneration |
| NF-κB | Nuclear factor kappa B |
| NLRP3 | NACHT, LRR and PYD domains-containing protein 3 |
| PDR | Proliferative diabetic retinopathy |
| PI3K | Phosphoinositide-3-kinase |
| PIGF | Placental growth factor |
| POS | Photoreceptor outer segments |
| RGC | Retinal ganglion cell |
| RGD | Arginyl-glycyl-aspartic acid |
| ROP | Retinopathy of prematurity |
| RPE | Retinal pigment epithelial |
| SMA | Smooth muscle actin |
| STZ | Streptozotocin |
| TGF | Transforming growth factor |
| TNF | Tumour necrosis factor |
| uPA | Urokinase-type plasminogen activator |
| VEGF | Vascular endothelial growth factor |

**Fig. 1.** Classification of the integrin receptor family.
known that RGD-binding integrins also bind to many other ECM proteins (e.g. vitronectin, fibrinogen and osteopontin) as well as growth factors, cytokines, enzymes, bacterial proteins and hormones (LaFoya et al., 2018; Ruoslahti, 1996; Wu and Reddy, 2012).

Integrin receptors are activated upon binding to their cognate ligands. The affinity and activation state can be influenced by engagement of intracellular proteins, such as talin and kindlin, to the integrin cytoplasmic domain. This outside-in and inside-out signalling enables integrins to 1) carry signals from the extracellular microenvironment and 2) respond to changes from inside the cell to induce a conformational change and modulate their affinity for extracellular ligands (Bouvard et al., 2013; Dalton et al., 2016; Klapholz and Brown, 2017). Integrins exist in a dynamic equilibrium between different conformations with varying ligand affinity: low, intermediate or high affinity states (see Fig. 2). Structural studies have revealed that integrins are inactive when their ectodomains are bent and this low affinity state is induced by intracellular binding of integrin inactivators, such as sharpin or mammary-derived growth inhibitor (MDGI; to the α-subunits) and ICAP-1 or filamin (to the β-subunits). During activation, integrins become extended and ligand binding can promote the transition from a closed intermediate to an open activated conformation of the headpiece. Integrins reach the open high affinity or fully activated state when they are simultaneously bound to the actin cytoskeleton as well as extracellular ligands (Banno and Ginsberg, 2008; Bouvard et al., 2013; Johansson and Mosher, 2013; Moser et al., 2009).

3. Retinal expression of integrins

A literature overview on the expression of integrin receptors and their subunits in the healthy retina is presented below and summarized in Table 1 and Fig. 3. Overall, these published findings indicate that RGD-binding integrins are expressed in all retinal layers.

3.1. Outer retinal distribution

Beside α2-, α5-, α4-, β1-, β2- and β3-integrin subunits (Brem et al., 1994; Vecino et al., 2015), mammalian retinal pigment epithelial (RPE) cells have been found to express αvβ3, αvβ5 and α5β1 integrins (Anderson et al., 1996; Chu and Grunwald, 1991; Elenner and Elenner, 1996; Li et al., 2009). In chick and rat retina, adhesion of RPE cells to Bruch’s membrane was found to involve an integrin-fibronectin interaction (Philp and Nachmias, 1987). The apical microvilli of the RPE cells expressed αvβ3 which is essential for phagocytosis and internalization of the shed POS-αvβ3 complex (Finnemann et al., 1997; Li et al., 2009; Lin and Clegg, 1998). Furthermore, the α3 subunit may play a role in the differentiation of rod photoreceptors during development via adhesion to the ECM component S-laminin which is present in the neural retina. Photoreceptor blocking antibodies to S-laminin profoundly reduced the appearance of cells that express rhodopsin in vitro, suggesting a role for S-laminin in the differentiation of rod photoreceptors (Hunter et al., 1992). In the outer nuclear layer, outer plexiform layer and photoreceptor layer of the human retina, immunolocalization has revealed expression of α2, α5, α4, α5- and β2-integrin subunits (Brem et al., 1994).

3.2. Inner retinal distribution

The α2-, α5-, α4-, α5- and β2-integrin subunits were observed in the human inner nuclear layer, inner plexiform layer (IPL) and retinal ganglion cell (RGC) layer (Brem et al., 1994; Elenner and Elenner, 1996). Immunohistochemistry performed on eyes from Sprague-Dawley rats demonstrated the presence of α2- and α5-integrin subunits, in addition to α1-, α2-, β1- and β3-integrin subunits, in the RGC layer, albeit with different intensities. Of these, integrin subunits α5 and β2 were also visible in the IPL (Vecino et al., 2015). Depending on the substrate on which cultured primary adult rat RGCs were grown, they expressed different integrins, which also affects their survival and capacity to

![Fig. 2. Schematic illustration of integrin activation.](image-url)
regenerate and extend neurites. More specifically, RGCs expressed $\alpha_1$, $\alpha_2$, $\alpha_5$, $\beta_1$ and $\beta_3$ when grown on laminin, $\alpha_1$ and $\beta_1$ when grown on collagen and $\alpha_5$, $\beta_1$ and $\beta_3$ when grown on fibronectin (Vecino et al., 2015). In situ hybridization revealed the presence of the RGD-binding integrin subunit $\beta_8$ in murine RGCs (Arnold et al., 2012).

### 3.3. Glial cells

In primate eyes, $\alpha_2^\gamma$, $\alpha_3^\gamma$, $\alpha_5^\gamma$, $\beta_1^\gamma$ and $\beta_4$-integrin subunits were localized to astrocytes within glial columns at the optic nerve head, suggesting participation in attachment of astrocytes to basement membranes (Morrison, 2006). Detailed analysis of integrin $\alpha_5^\gamma$- mice, which die at birth, revealed abnormalities in the laminar organization of the developing cortex and retina, indicating the importance of integrin-laminin interactions in proper development of the nervous system (Georges-Labouesse et al., 1998). Cultured human optic nerve head astrocytes were found to express $\alpha_3^\gamma$, $\alpha_4^\gamma$, $\alpha_5^\gamma$, $\alpha_7^\gamma$, $\beta_1^\gamma$, $\beta_2^\gamma$ and $\beta_5^\gamma$-integrin subunits (Neumann et al., 2014). The RGD-binding integrin subunits $\alpha_3$, $\beta_1$, $\beta_3$ and $\beta_8$ were found to be expressed by cultured human brain astrocytes. Cultured astrocytes derived from the developing murine retina expressed $\alpha_3$ and $\beta_8$ (Hirota et al., 2011), while in situ hybridization on the postnatal mouse retina revealed $\beta_8$ in Müller cells but not in astrocytes (Arnold et al., 2012). As described in human eyes by Brem et al. (1994), $\alpha_2$, $\alpha_3$, $\beta_1$- and $\beta_2$-integrin subunits were detected in the inner limiting membrane, which is formed by Müller cell end feet and astrocytes (Brem et al., 1994). The $\beta_1$-integrin subunit was proposed as surface marker for a novel magnetic-activated cell sorting-based approach of Müller cell enrichment from adult murine retinas, as it was expressed at significantly higher levels in Müller cells than retinal neurons (Grosche et al., 2016). Immunocytochemistry revealed expression of $\alpha_1$, $\alpha_2$, $\alpha_3$ and $\beta_1$-integrin subunits by Müller cells (Guidry et al., 2003). However, due to trans-differentiation of cultured Müller glia towards a fibroblast-like phenotype, expression of integrin subunits can shift and thereby not represent the healthy situation but rather their potential involvement in the generation of tractional forces (Guidry et al., 2003; Hauck et al., 2003). RGD-binding integrins $\alpha_5$ and $\alpha_6$ and their ligand vitronectin were expressed by astrocytes in vivo and were further upregulated during neurological diseases (Hirota et al., 2011; Milner et al., 1999; Yun et al., 2016). $\alpha_5$-integrin expression in astrocytes was found to be essential for neovascularization in the developing retina.

Mouse retinal microglia were found to express $\alpha_4$, $\alpha_5$, $\beta_1$, $\beta_2$, $\alpha_6$, $\beta_2$ and $\alpha_5$ (Milner and Campbell, 2003). Linked to their immunomodulatory function, microglial cells are also required for proper retinal blood vessel formation (Checchin et al., 2006). Although kindlin3 mainly binds and activates integrins (Meller et al., 2017), an integrin-independent action of kindlin3 was reported. It was demonstrated that vascular patterning at the ONL is controlled by microglial bipolarization via a kindlin3-regulated spatiotemporal control of TGF-$\beta_1$, independent of microglial integrins $\beta_1$ or $\beta_2$ (Duduki et al., 2020). The integrin $\alpha_5\beta_1$ plays a role in regulating the binding of microglia to laminins (Milner and Campbell, 2002). Biswas et al. (2017) showed that integrin $\beta_8$-mediated signalling is important in microglial activation via interaction with astrocyte-derived ECM, which might consequently contribute to pathobiology of retinal vasculature (Biswas et al., 2017).

### 3.4. Endothelial cells and pericytes

Vascular endothelial cells (ECs) and pericytes both expressed a subset of integrins: the RGD-binding integrin $\alpha_5\beta_1$, the leukocyte-specific integrins $\alpha_4$, $\alpha_5$, $\beta_1$, $\beta_3$ and $\beta_6$-integrin subunits (Neumann et al., 2014). The RGD-binding integrin subunits $\alpha_5$, $\beta_1$, $\beta_3$ and $\beta_8$ were found to be expressed by cultured human brain astrocytes. Cultured astrocytes derived from the developing murine retina expressed $\alpha_5$ and $\beta_8$ (Hirota et al., 2011), while in situ hybridization on the postnatal mouse retina revealed $\beta_8$ in Müller cells but not in astrocytes (Arnold et al., 2012). As described in human eyes by Brem et al. (1994), $\alpha_2$, $\alpha_3$, $\beta_1$- and $\beta_2$-integrin subunits were detected in the inner limiting membrane, which is formed by Müller cell end feet and astrocytes (Brem et al., 1994). The $\beta_1$-integrin subunit was proposed as surface marker for a novel magnetic-activated cell sorting-based approach of Müller cell enrichment from adult murine retinas, as it was expressed at significantly higher levels in Müller cells than retinal neurons (Grosche et al., 2016). Immunocytochemistry revealed expression of $\alpha_1$, $\alpha_2$, $\alpha_3$ and $\beta_1$-integrin subunits by Müller cells (Guidry et al., 2003). However, due to trans-differentiation of cultured Müller glia towards a fibroblast-like phenotype, expression of integrin subunits can shift and thereby not represent the healthy situation but rather their potential involvement in the generation of tractional forces (Guidry et al., 2003; Hauck et al., 2003). RGD-binding integrins $\alpha_5$ and $\alpha_6$ and their ligand vitronectin were expressed by astrocytes in vivo and were further upregulated during neurological diseases (Hirota et al., 2011; Milner et al., 1999; Yun et al., 2016). $\alpha_5$ integrin expression in astrocytes was found to be essential for neovascularization in the developing retina.

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### 4. RGD-binding integrins integrate multiple signalling cascades

Integrins can cross-talk with growth factors and their receptors, which has implications for the diversity of signalling responses occurring during normal physiology and pathogenesis (Eliceiri, 2001). The interaction between RGD-binding integrins and specific growth factors such as VEGF, angiopoietin (Ang), transforming growth factor-$\beta$ (TGF-$\beta$) and basic fibroblast growth factor (bFGF) has been extensively described.
in the literature. Since these growth factors have established important roles in retinal pathophysiology, the nature of their interactions with integrins is summarized below, as well as the link between RGD-binding integrins and various cytokines (e.g. interleukin-1β (IL-1β)) and enzymes (e.g. focal adhesion kinase (FAK)/proto-oncogene tyrosine-protein kinase Src (Spc), matrix metalloproteinases (MMPs)).

4.1. Growth factors

4.1.1. Vascular endothelial growth factor (VEGF) pathway

In mammals, the VEGF family comprises five family members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF) (Apte et al., 2019; Peach et al., 2018; Van Bergen et al., 2019). These members bind with different affinities to the VEGF receptors VEGFR-1, VEGFR-2 and VEGFR-3 and the VEGFR co-receptors neuropilin-1 and neuropilin-2 (Roy et al., 2006). VEGF-A (referred to as VEGF throughout this review) has been studied extensively and is well-known for its key role in (retinal/choroidal) angiogenesis and vascular permeability (Simons et al., 2016; Campochiaro and Akhlaq, 2020).

The VEGF/VEGFR pathway integrates with the RGD-binding integrins α5β1 and αvβ3 and serves to modulate signalling responses of both receptor systems (Mouloukova et al., 2017; Somanath et al., 2009). Several pharmacological studies reported a reduction in the mRNA levels of VEGF as well as VEGF-R2 (Chatterjee and Naik, 2012; Wilkinson-Berken et al., 2006), and a decrease in phosphorylation of VEGFR-2 by αβ3 and αβ5 neutralization (Mahabeswar et al., 2007; Santulli et al., 2008; Soldi et al., 1999; Tsou and Isik, 2001). In the context of the retina, it is described that cultured RPE cells under hypoxia will increase VEGF-levels, an event modulated through RGD-binding integrins (Douglass et al., 2006). For instance, a high Ang-2/Ang-1 ratio in combination with RGD-binding integrins is summarized below, as well as the link between RGD-binding integrins and various cytokines (e.g. interleukin-1β (IL-1β)) and enzymes (e.g. focal adhesion kinase (FAK)/proto-oncogene tyrosine-protein kinase Src (Src), matrix metalloproteinases (MMPs)).

4.1.2. Angiopoietin (Ang)/Tie pathway

Angiopoietins (Ang-1 and Ang-2) play an essential role in regulating vascular permeability, angiogenesis and inflammation (Eklund et al., 2017; Saharinen et al., 2017). Depending on the context, Ang-2 acts as an agonist or antagonist for the tyrosine kinase with immunoglobulin-like and EGF-like domains 2 or Tie2 receptor (Daly et al., 2006). For instance, a high Ang-2/Ang-1 ratio in combination with VEGF results in reduced vascular stability, leading to increased vascular leakage and neovascularization, while Ang-2 induces EC death and vessel regression in the absence of VEGF (Akwii et al., 2019; Hammes et al., 2011). While initially Ang-2 was reported to act as a competitive antagonist by binding, but not activating, Tie2 on ECs, Tie2 phosphorylation and thus activation by Ang-2 was also described, at least when present at high concentrations (Akwii et al., 2019; Daly et al., 2006). Likewise, Ang-1 fulfils a dual role in neovascularization. Several reports have described an anti-angiogenic and vascular stabilization function of Ang-1 in the eye (Hammes et al., 2011; Lee et al., 2014b; Nambu et al., 2004), whereas other studies reported the stimulating effect of Ang-1 in retinal as well as dermal and cerebral neovascularization (Cho et al., 2006; Hammes et al., 2011; Lee et al., 2013; Fang et al., 2018; Wang et al., 2019). Nevertheless, it is generally accepted that the Ang-2/Ang-1 ratio is augmented under pathological circumstances and results in vascular instability.

The Ang/Tie signalling pathway is modulated by its direct and indirect interactions with RGD-binding integrins. Hakanpaa et al. (2015) demonstrated that Ang-2-dependent activation of β3-integrin induced α5β1 translocation into the ends of actin stress fibres in ECs, resulting in vascular endothelium destabilization, which could be blocked by neutralization of β3-integrin. In addition, intravitreal (IVT) injection with α5 or β3-integrin inhibitors protected against Ang-2-dependent pericyte dropout (Park et al., 2014) and IVT treatment with neutralizing antibodies against α5β3 could attenuate Ang-2-induced astrocyte loss in the diabetic streptozotocin (STZ) mouse (Yun et al., 2016). On the other hand, Ang1 supplementation was suggested as potential therapy for ischemic vascular retinopathies as binding of Ang1 to αvβ3 was shown to induce FAK phosphorylation and fibronectin synthesis in retinal astrocytes, thereby stimulating guided reparative angiogenesis in the retina (Lee et al., 2013).

Both receptor homologues Tie1 and Tie2 can directly associate with the RGD-binding integrins αvβ3 and αvβ5 via their extracellular domains, and the integrin/Tie2 co-cluster stabilizes in the presence of fibronectin. Tie2 signalling was significantly sensitized to lower concentrations of Ang-1 following interaction with RGD-binding integrin co-receptors, whereas both a constitutive and transient interaction between Tie2 and αvβ3 following Ang-2 ligand binding was described (Cascone et al., 2005; Dalton et al., 2016; Thomas et al., 2010). Ang-2-induced Tie2/αvβ3 receptor complex formation could recruit and activate FAK, followed by focal adhesion dissociation as well as internalization and lysosomal degradation of integrin α5β1, thereby causing EC destabilization (Thomas et al., 2010). Recently, Miranda et al. (2019) indicated that α5β1 integrin heterodimers sequester Tie2 at non-junctional locations in the EC membrane. Upon treatment with AXTI107, the heterodimer is disrupted, leading to translocation and complex formation of α5 and Tie2 at EC-EC junctions and to conversion of Ang2 into a strong Tie2 agonist, whereby EC-EC contacts are reinforced and monolayer permeability is reduced (Mirando et al., 2019).

Besides their capacity to bind Tie receptors, direct binding of Ang ligands to RGD-binding integrins has also been demonstrated in Tie2-low ECs and several cell types lacking Tie2 such as cancer cells, neurons and cardiomyocytes (Carlson et al., 2001; Dalton et al., 2016; Felcht et al., 2012; Lee et al., 2014b). Although Ang-2 was reported to bind directly to the RGD-binding integrins αvβ3, αvβ5 and α5β1 by ELISA (albeit with low affinity), thereby promoting Tie2-independent neovascularization (Felcht et al., 2012; Hakanpaa et al., 2015), these results should be interpreted with caution since demonstrating this interaction required the use of a covalent cross-linking agent. While Ang-2 was demonstrated to activate α5β1-integrin via its N-terminal domain (Hakanpaa et al., 2015) and the Glutamine Gln-362 residue of Ang-2 (Lee et al., 2014a), the fibrinogen-like receptor binding domain of Ang-1 was essential for direct interaction with αvβ3 and αvβ5 in a Tie2-independent manner (Dallabrida et al., 2008; Dalton et al., 2016).
4.1.3. Transforming growth factor-β (TGF-β)

In physiological conditions, the transforming growth factor-β (TGF-β) subfamily, consisting of TGF-β1, -2 and -3, is expressed at low levels and involved in for instance cell growth and matrix synthesis. In contrast, in pathological conditions, these growth factors are expressed at high levels and may cause accumulation of matrix components, fibrosis, inflammation and immune dysregulation (Finnson et al., 2020; Wan and Flavell, 2007). TGF-β is expressed as a full-length protein, containing a large N-terminal domain (referred to as latency-associated peptide LAP-1, 2 or 3) and a smaller C-terminal domain (referred to as mature TGF-β, which can be separated by intracellular proteolysis). A dimer of LAP can form a non-covalent complex with a dimer of mature TGF-β, called LAP- or latent-TGF-β. This complex will remain in the cell until it is bound by latent TGF-β binding proteins (LTBPs) -1,-2,-3 or -4 to form a large latent complex (LLC). This LLC is secreted from cells and needs further processing to release TGF-β from LAP, which can then bind and activate TGF-β receptors (Annes et al., 2003; Lawrence, 2001; Miyazono et al., 1991; Rifkin, 2005; Taipale et al., 1994; Taylor, 2009).

All α-containing RGD-binding integrins have been shown in vitro to release and thus activate TGF-β1 or -3, more specifically via interaction or binding with the RGD motif in the LAP-1 and -3 peptide, respectively (Annes et al., 2003; Asano et al., 2005; Mu et al., 2002; Munger et al., 1999; Reed et al., 2015). The contribution of α-containing RGD-binding integrins to TGF-β1 activation was further assessed in knock-out animals containing a mutation in the TGF-β1 gene encoding a non-functional or inactive variant of LAP’s integrin binding site (RGE instead of RGD). These mice demonstrated the characteristic phenotype of TGF-β1 knock-out animals (e.g. inflammation and vascular defects), while a normal production of latent TGF-β1 was present (Yang et al., 2007b). On the other hand, upon activation and receptor engagement, TGF-β1 or -3 signalling can also induce the expression of α-containing integrins (Honda et al., 2010; Zambruno et al., 1995).

The first integrin identified as a LAP-TGF-β activator was αvβ3 (Munger et al., 1999), requiring an intact integrin cytoplasmic domain and the presence of other ECM proteins (e.g. fibronectin) (Guerrero and McCarty, 2018). This RGD-binding integrin can play a very important role in the development of fibrotic conditions (Basta et al., 2020; Horan et al., 2008; Huang et al., 1996; Munger et al., 1999; Puthawala et al., 2008; Wang et al., 2007) and pathological angiogenesis (Guerrero and McCarty, 2018), although its role in the eye remains undefined. Another integrin that was described to activate TGF-β is αvβ6, expressed by normal epithelial and neuronal cells in vivo (Araya et al., 2006; Mu et al., 2002). In contrast to αvβ3 activation by αvβ6 does not require the integrin cytoplasmic domain, but rather the presence of membrane type 1-matrix metalloproteinase (MT1-MMP) on the cell surface or in the ECM (Arnold et al., 2012). In the retina, it has been described that deletion of αvβ6 in astrocytes led to impaired blood vessel sprouting and hemorrhages, a phenotype that correlated with reduced ECM-binding latent TGF-β (Hirota et al., 2011), indicating an essential role for αvβ6 in neovascularization in the developing retina. Moreover, deletion of the β6-subunit led to impaired vascular development in the retina, especially in the deep vascular plexus, which was similar to what was observed after retinal deletion of TGF-β1. The observed reduction in phospho-SMAD3 levels in ECs after retinal deletion of integrin β6 supported αvβ6-mediated TGF-β signalling in ECs in vivo (Arnold et al., 2012; Roy et al., 2006). Finally, a few reports also described a potential role for αvβ3 (Song et al., 2016) and αvβ6 (Shovmick et al., 2009; Munger et al., 1999) in the activation of TGF-β, whereas the exact role of the RGD-binding integrins αvβ3 and αvβ6 in activating latent TGF-β remains controversial (Araya et al., 2006; Asano et al., 2005; Li et al., 2013; Thibault et al., 2001; Wipff et al., 2007).

4.1.4. Basic fibroblast growth factor (bFGF)

bFGF (also known as FGF2) is a cell signalling protein that is involved in a wide variety of processes and is especially relevant to ocular angiogenesis. Many studies have demonstrated a link between RGD-binding integrins and bFGF-induced neovascularization, from which αvβ3 and αvβ6 are the most described. Different studies reported that αvβ3 expression (and not αvβ6) in ECs of corneal and chick chorioallantoic membrane (CAM) assays was significantly increased upon induction of angiogenesis using bFGF (Brooks et al., 1994; Friedlander et al., 1995; Koch and Distler, 2007). The exact mechanisms underlying bFGF-induced ocular angiogenesis dependent on integrins are unclear, although one research group reported a direct binding of αvβ3 to bFGF (Mori et al., 2008). The pro-angiogenic features induced by bFGF could be significantly reduced in vitro or ex vivo by using specific anti-αvβ3 antibodies (Brooks et al., 1994; Runnait et al., 1997), by a RGD peptide-mimetic cyclo[DKP-RGD] 1, which showed low nanomolar affinity for αvβ3 and αvβ5 (Fanelli et al., 2014), by the αvβ6 inhibiting molecule JSM6427 (Maier et al., 2007) or by T3RI265, which suppresses αvβ3-MMP-2 interactions (Silletti et al., 2001), whereas the use of an anti-αvβ3 antibody had no effect (Friedlander et al., 1995; Koch and Distler, 2007). Besides αvβ3, the integrin αvβ6 has also been demonstrated to be highly upregulated in the CAM-model after bFGF-induced angiogenesis (Kim et al., 2000a), which requires interaction of this receptor with fibronectin (Aota et al., 1994). Inhibition of this RGD-binding integrin reduced vascular leakage in a rabbit VEGF/bFGF pellet-induced model of choroidal neovascularization (CVN) (Zahn et al., 2009). Moreover, it was described that blocking tube formation in an bFGF-induced fibroin exudate, containing fibrin and fibrinogen, requires the simultaneous inhibition of αvβ5- and αvβ3-integrins (Laurens et al., 2009). On the other hand, a recent report described that integrin αv suppression efficiently prevented the production of TGF-β and bFGF, but not VEGF, showing a direct effect of αv inhibition on the expression of bFGF (Lv et al., 2020). The integrins αvβ3 and αvβ6 can thus play a key role in bFGF-induced angiogenesis, which is in contrast to VEGF-induced neovascularization, where mainly αvβ3 is involved (Friedlander et al., 1995). Indeed, different integrins are involved in EC-mediated angiogenesis, depending on the specific growth factor that is released by these cells.

4.1.5. Other growth factors

In addition to the more thoroughly investigated interactions between RGD-binding integrins and VEGF, Ang, TGF-β and bFGF, there is also (limited) information available on the interplay between RGD-binding integrins and PI GF (Hoffmann et al., 2013; Pipp et al., 2003), epidermal growth factor (EGF) (Caswell et al., 2008; Moro et al., 1998; Suzuki et al., 2010; Vella et al., 2003), insulin-like growth factor binding proteins (IGFBPs) (Brandt et al., 2015; Feng et al., 2015; Jones et al., 1993; Wang et al., 2006), platelet-derived growth factor (PDGF) (Ding et al., 2003; Jester et al., 2002; Liu et al., 2001; Moteigi et al., 2011; Schneller et al., 1997; Woodard et al., 1998), and connective tissue growth factor (CTGF) (Chen et al., 2004; Gao and Briggstock, 2006; Hennig et al., 2016; Kiwanuka et al., 2013; Lipson et al., 2012), all in the context of angiogenesis and/or fibrosis. Finally, αvβ3 and αvβ6 interactions with angiotensin-like proteins (ANGPTLs) were observed. Whereas binding of ANGPTL2 to αvβ3 was found to promote inflammation (Takano et al., 2019) (see also section 5.1.2), the ANGPTL4 - αvβ3 interaction seemed necessary for inducing protective effects against hypoxia-induced permeability (e.g. increased tight junction integrity) in the retina, more specifically by modulating the Src signalling pathway downstream of VEGFR-2. This observation indicates that the activation of the ANGPTL4 - αvβ3 axis might be a potential protective pharmacetical intervention in pathological retinal conditions (Gomez et al., 2016).

While interactions between various growth factors and αvβ3, αvβ6 and αvβ1 integrins have been extensively described, their specific role in the eye or retina remains ill-defined. Nevertheless, the strong association between RGD-binding integrins and key growth factors is an essential pathway in pathological angiogenesis, inflammation, vascular leakage and fibrotic reactions and this makes them very relevant for retinal diseases.
4.2. Cytokines and enzymes

4.2.1. Cytokines

Cross-talk between integrins and cytokines (e.g. IL-1β, IL-6 and tumour necrosis factor alpha (TNF-α)) can regulate a range of biological mechanisms including inflammation and angiogenesis (Takada et al., 2017; Wang et al., 1997; Zhu et al., 1998). Although the role of RGD-binding integrins (e.g. α5β1, αvβ3, αvβ6 and α9β1) in IL-1β-mediated signalling is described in various non-ocular disease models (Auron et al., 1984; Gaunter et al., 2008; Peng et al., 2005), only one report studied the role of α5β1 and IL-1β on RPE cells in the context of dry AMD. Inflammamsose-dependent IL-1β and monocyte chemotactic protein (MCP-1) release from human RPE cells was dependent on α5β1 and α9β2, which could be an important mechanism in this age-related maculopathy (Bian et al., 2018). Another RGD-binding integrin, αvβ3, has also been described to regulate IL-1β-dependent macrophage inflammatory responses (Antonov et al., 2011) and TNF-α induced angiogenic processes (Ogawara et al., 2006), both via mechanisms dependent on nuclear factor-kappa B (NF-κB) activation. In addition, αvβ3 expression was upregulated in TNF-α-induced models of angiogenesis (Brooks et al., 1994; Friedlander et al., 1995; Koch and Distler, 2007), indicating that the interaction of αvβ3 and this cytokine is important in a diverse range of signalling events. The use of several general RGD-binding integrin inhibitors completely inhibited TNF-α and IL-6 increase upon LPS stimulation of macrophages in vitro (Hsu et al., 2011; Miranda et al., 2020; Monick et al., 2002; Zaveri et al., 2014) and in vivo (Ding et al., 2015; Miranda et al., 2020).

4.2.2. FAK/Src tyrosine kinases

The tyrosine kinases FAK and Src are important mediators of integrins, regulating cytoskeletal organization and motility in response to cell adhesion (Shattil, 2005). Integrin-mediated adhesion induces auto-phosphorylation of FAK, thereby creating a binding site for Src. Several key integrins (e.g. α5β1 and α5β2) seemed to be mediated by the activation of the integrin αvβ3, FAK, PI3K/Akt and IκB kinase (IκBκ) in chorioretinal vascular ECs (You et al., 2014), suggesting that these pathways can be considered as a potential inhibitory target for DR. Direct interaction between integrins and Src in the retina was only described during neurite outgrowth on primary retinal neurons, in which activation of Ephrin-A5 was dependent on the activation of β1-integrins and various members of the Src family (Davy and Robbins, 2000).

4.2.3. Matrix metalloproteinases (MMPs)

The relationship between integrins and matrix metalloproteinases (MMPs) is extensively described in literature but remains complex and context-dependent. There is evidence that fibronectin, through its interaction with RGD-binding integrin receptors, can regulate the expression and activity of MMPs, especially MMP-2 and MMP-9 (Hultala et al., 1995; Shibata et al., 1997; Stanton et al., 1998; Xie et al., 1998). Several papers also described a link between increased MMP levels and integrin activation, leading to altered cell adhesion, growth and motility as demonstrated for integrin αvβ3 (Kanda et al., 2000; Cohen et al., 2014; Crisp et al., 2014), for αvβ8 (Mu et al., 2002) and for α5β2 (Bax et al., 2004; Hultala et al., 1995). In the eye, MMP-2 inhibition (but not MMP-9 inhibition) was shown to reduce axon outgrowth of mouse RGCs via a β1-integrin dependent pathway, using ex vivo retinal explant models (Gaublomme et al., 2014). Moreover, increased RPE cell adhesion to collagen matrix (Ulbrich et al., 2011), diabetes-induced pericyte cell death (Yang et al., 2007a) and increased Müller cell migration (Lorenz et al., 2015) were related to increased αv or β1-integrin and MMP-2 expression, which are key processes in AMD and DR. On the other hand, there is also evidence that activation of MMPs can lead to loss of integrins. Indeed, in various retinal neurodegeneration models, upregulated MMP-9 levels were associated with decreased RGC expression of β1-integrin and FAK and Akt dephosphorylation, all prior RGC apoptosis. Cell death was prevented by maintaining β1-integrin activation with agonistic antibodies (D’Onofrio et al., 2018; Santos et al., 2012). All these results point towards a clear, but complex, link between integrins and MMP. The interaction is context-dependent and will determine whether activated MMPs will lead to either loss or activation of integrins and whether RGD-binding integrin receptors will regulate the expression and activity of MMPs.

Overall, a complex interplay between integrins and cytokines, as well as various enzymes is described mainly in the context of inflammation (e.g. α5β1 and α9β2), angiogenesis/edema (α5β2), RPE cell phagocytosis (α5β2) and/or RGC apoptosis (β1-integrins). Although the relevance of these interactions on an ocular level is not described that much in detail, these could possibly play a role in various retinopathies, such as AMD and DR.

To summarize, RGD-binding integrins affect a multitude of disease-related proteins (e.g. growth factors, cytokines and enzymes) and their molecular pathways, and are able to modulate growth factor signalling in various ways (see also Fig. 4). Given their potential to integrate multiple cellular signalling networks, RGD-binding integrin antagonists are assumed to exert broader biological effects and target additional points in pathological processes as compared to anti-VEGF therapies.

5. Role of RGD-binding integrins in disease hallmarks of diabetic retinopathy and neovascular age-related macular degeneration

Although DR and nvAMD are distinct retinal disorders, they share several common disease hallmarks such as chronic inflammation, vascular leakage, neovascularization and fibrosis. In the following paragraphs, we will discuss the molecular interactions and signalling pathways by which RGD-binding integrins regulate these pathophysiological processes. The cellular and molecular machinery by which RGD-binding integrins contribute to these vision-threatening processes has been derived mostly from cell culture observations. However, the importance of RGD-binding integrins has also been studied in experimental animal models for DR and nvAMD and these have proved valuable for advancing our understanding, not least in terms of the potential for therapeutic targeting of these receptors (see also Table 2).

5.1. Inflammation

As discussed in section 4.2.1, RGD-binding integrins can regulate inflammatory processes by interacting with several cytokine-mediated signalling pathways. This section will review the current understanding on the role of RGD-binding integrins in leukocyte adhesion, migration and infiltration at the vascular endothelial barrier and in
modulating inflammatory immune signalling pathways.

5.1.1. Leukocyte-endothelial interactions

Blocking integrin function or genetic deletion of integrins has been described to interfere with the adhesion and transmigration of circulating leukocytes to/through the ECs of the blood vessel wall, which are critical events during inflammatory reactions and various vascular processes. Leukostasis likely contributes to retinal vascular leakage and thereby enhances the pathogenic effects on the retina during DR. The leukocyte adhesion cascade involves distinct adhesive leukocyte-EC interactions and signalling pathways in a specific spatiotemporal manner. The initial interaction between leukocytes and endothelium appears to be transient, resulting in the rolling of leukocytes along the vessel wall. Subsequently, leukocytes become activated by endothelial factors, leading to their arrest and firm adhesion, and will ultimately, transmigrate through the intercellular junctions into the underlying tissue, also called diapedesis. Once integrins are activated on the leukocyte surface, they mediate strong adhesive interactions with counter-receptors (ligands) on the endothelium, including vascular cell adhesion molecule-1 (VCAM-1 or CD106) and intercellular adhesion molecule-1 (ICAM-1 or CD54), which are highly upregulated in response to inflammatory mediators such as TNF-α (Barouch et al., 2000; Goda et al., 2000; Gustavsson et al., 2010; Jin et al., 2006; Muller, 2003; Weerasinghe et al., 1998).

Fig. 3. Integrin expression profile in the retinal cells.

Fig. 4. RGD integrin receptors integrate multiple pathological signalling cascades through their influence on a broad range of disease-related proteins and their modulation of growth factor signalling at multiple levels.
Table 2
Overview of preclinical studies using RGD integrin receptor antagonists in animal models for DR, DME or nAMD.

| Compound | Company | RGD- integrin receptor subtype target | Effect | Model/species | References |
|----------|---------|--------------------------------------|--------|---------------|------------|
| Risuteganib (ALG-1001) | Allegro Ophthalmics | αιβ2, αιβ3, αιβ5, αιβ6, αιβ5 | Reduced leakage and angiogenesis | Mouse laser-induced CNV and ROP model Mouse hVEGF transgenic model | (Boyer, 2012; Kaiser et al., 2013) |
| THR-687 | Oxurion NV | αιβ2, αιβ5, αιβ1 | Reduced leakage and fibrosis | Cyno laser-induced CNV model Mouse VEGF-induced model | Hu et al. (2019) |
| AXT107 | AsclepiX Therapeutics | αιβ3, αιβ1 | Reduced leakage, neovascularization and inflammation | Mouse laser-induced CNV and ROP model Rabbit VEGF-induced leakage model Mouse LPS- and TNF-α-induced model Transgenic Rho/VEGF Tet/opsin/VEGF and IRBP- rTA/TRE-Ang2 mice | (Mirando et al., 2019, 2020; Silva et al., 2017) |
| OTT-166 (formerly SF-0166) | OcuTerra Therapeutics (formerly SciFuier Life Sciences, Inc) | αιβ2, αιβ4, αιβ6 | Reduced leakage and neovascularization | Mouse laser-induced CNV and ROP model | Aikew et al. (2018) |
| AS101 | Volociximab | αιβ3, αιβ4, αιβ1 | Reduced neovascularization | Reduced leakage | (Feramenda Biopharmaceutical) Ramakrishnan et al. (2006) |
| JSM6427 | Jerini AG & Shire Pharmaceuticals | αιβ1 | Reduced leakage, neovascularization and inflammation | Cyno laser-induced CNV model | (Maier et al., 2007; Umeda et al., 2006; Zahn et al., 2009) |
| OCU-200 | Ocugen | αιβ3 | Reduced leakage and neovascularization | Mouse laser-induced CNV and ROP model | (Ocugen) |
| SB-267268 | GlaxoSmithKline | αιβ2, αιβ5 | Reduced neovascularization | Reduced leakage | Wilkinson-Berka et al. (2006) |
| JNJ-2676713 | J&J Pharmaceuticals | αιβ2, αιβ5 | Reduced leakage, neovascularization and inflammation | Mouse ROP model Diabetic rat STZ model | Santulli et al. (2008) |
| Lebecetin | SATT Lutech | αιβ1, αιβ- integrins | Reduced neovascularization | Mouse laser-induced CNV and ROP model | Montassar et al. (2017) |
| EGT022 | EyeGene | RGD-containing disintegrin | Reduced leakage | Mouse ROP model | Jang et al. (2016) |
| C16Y | Odin Biotech Partners | αιβ3, αιβ1 | Reduced neovascularization | Rat laser-induced CNV model | Kim and Casy (2010) |
| ATN-161 | / | αιβ1 | Reduced leakage, neovascularization and inflammation | Rat/mouse laser-induced CNV and ROP model Transgenic Tet/opsin/VEGF mice | Sui et al., 2018a, 2018b; Wang et al., 2011, 2016 |
| Tetraisodothyroacetic acid (tetrac) | / | αιβ3 | Reduced neovascularization | Mouse ROP model | Yoshida et al. (2012) |
| GOPPP | / | αιβ3 | Reduced neovascularization | Mouse ROP model | Li et al. (2014) |
| TAT PTD-endostatin-RGD | / | αιβ3 | Reduced neovascularization | Mouse ROP model | Li et al. (2016) |

bFGF: basic fibroblast growth factor; CNV: choroidal neovascularization; RGD: Arginyl-glycyl-aspartic acid; ROP: retinopathy of prematurity; STZ: streptozotocin; VEGF: vascular endothelial growth factor.

The leukocyte adhesion receptors such as αιβ2, αιβ5 and αιβ1-integrins are well-known players in this leukostasis process (Mitroulis et al., 2015). Moreover, under inflammatory conditions, antibodies against the RGD-binding integrin αιβ2 were able to inhibit trans-endothelial monocyte migration to a similar extent as anti-β1 or anti-β2 antibodies, likely by modulating the αιβ2 integrin-mediated migration of monocytes on ICAM-1 (Weerasinghe et al., 1998). The presence of β1-and β3-integrins in close association with cell surface tissue transglutaminase on podosome-like adhesive structures of differentiated monocytes further supports the involvement of RGD-binding integrins in extravasation and migration of leukocytes during inflammation (Akimov and Belkin, 2001). The RGD-binding integrin αιβ3 was also described to regulate lymphocyte migration and subsequent extravasation on/through ECs and interstitial tissue, which seems to be modulated by a cross-talk between αιβ3 and αιβ1 integrins (Imhof et al., 1997; Lacy-Hulbert et al., 2007). In addition, myeloid cell transmigration can result from engagement of integrin-associated protein (IAP or CD47) - αιβ3 on ECs by leukocyte platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31), leading to increased intracellular Ca2+ levels, EC retraction and loss of tight junctions (Porter and Hogg, 1998). Adhesion and trans-endothelial leukocyte trafficking are also modulated by αιβ1. This effect was suggested to result from its close physical and functional interaction with endothelial endoglin, enhanced αιβ1-fibronectin binding and/or FAK-extracellular signal-regulated kinase (ERK) signal pathway activation (Labus et al., 2018; Rossi et al., 2013; Yang et al., 2012). Trans-endothelial migration of neutrophils was regulated by combined αιβ3 and αιβ1 integrin activity (Gonzalez et al., 2007).

Besides these in vitro studies, there is limited in vivo evidence that points towards a role for RGD-binding integrins in leukocyte-endothelial
interactions. Subcutaneous administration of the peptidomimetic $\alpha\beta$-integrin antagonist S247 (25 mg/kg body weight, twice/day) in an acute kidney allograft rejection model could significantly reduce mononuclear cell (T-cells, monocytes, macrophages) infiltration by approximately 45%, adhesion by 65% and trans-endothelial migration by 60% (Bedke et al., 2007). Concomitant with the impaired adhesion and migration of T-cells in the absence of $\beta3$ in vitro, lymphocyte infiltration was also significantly attenuated (60% reduction for CD4 T-cells, 36% for CD8 T-cells) in a model of heart transplant rejection using $\beta3$ knock-out animals (Lacy-Hulbert et al., 2007). On the other hand, systemic treatment with $\alpha\beta3$ blocking antibodies (4 mg/kg) in a sepsis-induced experimental animal model could decrease, yet not completely prevent, mortality, but could not reduce sepsis-induced cytokine/chemokine serum levels. In addition, thioglycollate-induced leukocyte migration into the peritoneum could not be inhibited by $\alpha\beta3$ neutralization (Su et al., 2013). Although this study suggested $\alpha\beta3$ as a regulator of endothelial permeability by affecting inflammation-induced EC cytoskeletal rearrangement, additional molecular players are likely to contribute to the inflammation-driven processes in these models of severe disease. However, differences in for instance dosages, tissues and thus expression of integrins might explain the discrepancy between these in vivo studies.

The in vivo effect of RGD-binding integrins on leukocyte adhesion in the eye is to our knowledge poorly investigated. While retinal vascular permeability was significantly inhibited, leukocyte adhesion was (not-significantly) reduced by 48% after oral administration of the $\alpha\beta1$-integrin antagonist JNJ-26076713 (60 mg/kg body weight, twice/day) in STZ-induced diabetic rats (Santulli et al., 2008). Intravitreal pretreatment with the $\alpha\beta3$ and $\alpha\beta1$ inhibitor AXT107 (1 $\mu$g/eye) in the acute TNF-$\alpha$-induced mouse model significantly reduced the number of retinal adherent leukocytes by 29% (Mirando et al., 2020). Of note, AXT107 was found to reduce TNF-$\alpha$-induced VCAM-1 and ICAM-1 levels on HUVECs (Mirando et al., 2020).

### 5.1.2. Leukocyte activation

Next to their implication in immune cell recruitment, $\alpha\beta3$ and $\alpha\beta1$ activation also regulates macrophage inflammatory immune signalling pathways at the site of injury. These integrin-mediated processes are mainly driven via NF-$\kappa$B, a transcription factor of a wide variety of genes coordinating inflammatory responses, including TNF-$\alpha$, IL-1$\beta$ and IL-6 (Antonov et al., 2011; Kurihara et al., 2011; Liu et al., 2017) (see also section 4.2 and Fig. 5). As such, $\alpha\beta3$ was demonstrated to enhance TNF-$\alpha$- and LPS-induced macrophage-related inflammatory responses by inducing NF-$\kappa$B activation (Antonov et al., 2011). The PI3K/Akt signalling pathway was postulated as possible mechanism by which $\alpha\beta3$ activation triggers NF-$\kappa$B dependent pro-inflammatory gene activation. Integrin $\alpha\beta3$ blockade also partially reduced cell adhesion and TNF-$\alpha$ production of phorbol myristate acetate (PMA)-activated monocytes/macrophages, which was mediated via protein kinase C (PKC) and ERK signalling cascades, known upstream activators of NF-$\kappa$B in macrophages (Chen and Lin, 2001; Kurihara et al., 2011). LPS-induced expression of the pro-inflammatory cytokines TNF-$\alpha$ and IL-6 in the mouse macrophage cell line RAW264.7 was suppressed by inhibiting $\alpha\beta1$ (Kanda et al., 2012). Interestingly, $\alpha\beta1$ was identified as receptor for ANGPTL2, which has been repeatedly described to mediate acute and chronic inflammatory processes in the body, including the eye (Aoi et al., 2011; Hirasa et al., 2016; Kanda et al., 2012; Tabata et al., 2009; Thorin-Trescases and Thorin, 2017). Binding of ANGPTL2 to $\alpha\beta1$ initiates an inflammatory cascade in macrophages and vascular ECs, mediated via NF-$\kappa$B activation (Takano et al., 2019). Moreover, retinal leukocyte adhesion and infiltration, as well as expression of MCP-1, I. Van Hove et al.
ICAM-1, IL-6, TNF-α and NF-κB p65 subunit, were reduced in ANGPTL2 knock-out mice (Kanda et al., 2012). According to Hirasawa et al. (2016), ANGPTL2-induced inflammatory responses are, at least in part, mediated via integrin α4 and/or β2. Although, Umikawa et al. (2015) could not observe inhibition of binding of ANGPTL2 to human monocytic THP-1 cells by α4- or β3-neutralizing antibodies, indicating cell type-specific responses. However, Hirasawa et al. (2016) observed that application of an anti-integrin αβ2 inhibitor in RAW264.7 cells did not suppress ANGPTL2-induced mRNA expression of the inflammatory mediators IL-6, MMP-9 and TGF-β, except for IL-1β. This latter finding is in accordance with the observation that αβ2 induces IL-1β transcription in differentiated human monocytes after direct contact with the bacterial surface protein Td92. Moreover, treatment of these cells with a neutralizing αβ1 antibody reduced NACH, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome expression, caspase-1 activity and IL-1β secretion (Jun et al., 2012). The NLRP3 inflammasome is a multiprotein complex involved in innate immunity and suggested to play an important role in retinal pathology (Chaurasia et al., 2018; Sui et al., 2018; Tarallo et al., 2012; Van Hove et al., 2020). Upon activation, NLRP3 inflammasome triggers caspase-1 activity, which then cleaves and activates the pro-inflammatory cytokines IL-1β and IL-18. Blocking αβ2 in an experimental stroke model resulted in decreased IL-1β levels and leukocyte infiltration as well as a reduction in infarct volume, functional deficits, blood-brain barrier (BBB) permeability and edema (Edwards et al., 2019; Roberts et al., 2017). Next to ANGPTL2, also Ang-1 and Ang-2 are known activators of αβ1 previously mentioned in section 4.1.2 (Imanishi et al., 2007; Felcht et al., 2012). Interestingly, in macrophages and microglia, αβ1 was identified as the main receptor for Ang-2, over Tie2. Moreover, recent findings indicate that Ang-2 signalling via αβ1 enhances pro-inflammatory M1 polarization of macrophages/microglia during neuro-inflammation (Lee et al., 2018; Li et al., 2020). Treatment with α4- or β3-integrin blocking antibodies reversed microglia activation, i.e. from amoeboid to ramified morphology, when grown on vitronectin or fibronectin respectively, which was demonstrated to rely on pro-MMP-9 expression (Milner et al., 2007). Additional function-blocking experiments, together with the previously described expression of αβ1 and αβ3 in microglia, indicate both RGD-binding integrins as important mediators in microglia activation (Milner et al., 2007). Of note, pro-as well as anti-inflammatory functions for αβ3 integrins have been described in different animal studies. While β3 deficiency, α4, and αβ3 inhibitors suppressed inflammatory responses in animal models for lung injury, acute kidney allograft rejection and heart transplant rejection, respectively, anti-inflammatory effects for αβ3 were observed in mouse models of atherosclerosis (Bedke et al., 2007; Lacy-Hulbert et al., 2007; Lee et al., 2007; Moon et al., 2009; Schneider et al., 2007; Weng et al., 2003). In addition, β3 knock-out animals subjected to experimental sepsis showed greater lethality and increased systemic vascular leakage, suggesting anti-inflammatory functions for αβ3 (Su et al., 2012).

Blockage of inflammatory reactions in the eye has been demonstrated after αβ1 neutralization. As such, inhibition of αβ1 by the small peptide ATN-161 could reduce VEGF-induced retinal leakage and neo-vascularization by suppressing NLRP3 inflammasome signalling and was shown to inhibit NF-κB activation and suppress retinal neo-vascularization in the mouse retinaopathy of prematurity (ROP) model (Sui et al., 2018a, 2018b). In addition, intravitreal administration of the αβ1 inhibitor JSM6427 in a rabbit model of retinal detachment resulted in a significant inhibition of microglial/macroglial proliferation (Zahn et al., 2010). Subconjunctival or topical application of the small molecule integrin αβ1 inhibitor CLT-28643 significantly reduced leukocyte density in the bleb in a mouse model of glaucoma filtration surgery (Van Bergen et al., 2016).

To conclude, αβ3 and αβ1 are the RGD-binding integrin receptors which have been linked regularly to inflammatory responses that are central to the pathology of retinal vascular disorders, especially by mediating immune cell interactions with ECs and the ECM and by inducing inflammatory signalling pathways.

5.2. Vascular leakage

Several papers have reported on the role of RGD-binding integrins αβ3, αβ7 and αβ1 in vascular leakage, as demonstrated in mice carrying a genetic deletion in an integrin subunit or by functional inhibition of integrins (Hakanpaa et al., 2018; Elieciei et al., 2002; Robinson et al., 2004; Su et al., 2012). Remarkably, while vascular permeability was reduced in β3 and β3-deficient animals or after αβ3 and β1 neutralization, vascular leakage was either unaltered or increased in β3 knock-out mice or after αβ3 blockade, indicating distinct regulatory functions for αβ3, αβ7 and αβ1 on endothelial barrier permeability.

In the brain, the RGD-binding integrins αβ1, αβ3, αβ7 and αβ8 have been associated with BBB permeability during pathogenesis (Edwards et al., 2019; Edwards and Bix, 2019; Lee et al., 2019; Roberts et al., 2017; Shimamura et al., 2006; Wang et al., 2019, 2020). Similarly, in the eye, several publications reported on the role of the RGD-binding integrins αβ1, αβ2 and αβ5 in retinal and choroidal vascular permeability. Inhibition of αβ2 integrin by volociximab or JSM6427 significantly inhibited angiogenesis-driven leakage in the laser-induced CNV cynomolgus monkey model (Ramakrishnan et al., 2006; Zahn et al., 2009). In addition, choroidal vascular leakage was dose-dependently suppressed by JSM6427 in a VEGF/BFGF-induced CNV rabbit model (Zahn et al., 2009). Treatment with the αβ2 and αβ3 integrin-binding peptide AXT107 significantly reduced VEGF-induced retinal leakage in the rabbit eye and in rhesus monkeys, concomitant with disruption of VEGF, hepatocyte growth factor (HGF) and PGDF-BB signalling as demonstrated using cultured human retinal ECs (Silva et al., 2017). More recently, application of AXT107 was shown to suppress Ang-2, LPS and TNF-α-induced vascular leakage in the mouse eye (Mirando et al., 2019, 2020). In detail, AXT107 was found to dissociate α3 from the αβ3 complex at non-junctional sites of ECs, leading to the translocation and complex formation of α3 and Tie2 at EC junctions, resulting in activation of Akt-mediated survival pathways, reduced myosin regulatory light chain 2 (MLC-2) activity, actin rearrangement and thus stabilization of cell-cell interactions.

Topical ocular application of the αβ1 antagonist SF1066 dose-dependently attenuated retinal vascular permeability in the rabbit VEGF-induced leakage model (Askew et al., 2018). On the contrary, inhibition of hypoxia-induced retinal vascular permeability by ANGPTL4 was found to rely on the binding of ANGPTL4 to αβ3, indicative for a protective effect of αβ3 on permeability. The vaso-protective effects of ANGPTL4/αβ3 on adherens and tight junctions were ascribed to modulation of the VEGFR-2/Src signalling pathway (Gomez et al., 2016). In addition, vascular leakage was augmented in ANGPTL4 knockout mice subjected to laser-induced CNV (Gomez et al., 2016). Blocking α4 integrins via cyclic RGD peptide or JNJ-26076713 administration was demonstrated to reduce retinal vascular permeability in the rat laser-induced CNV model and in the rat STZ-diabetes model, respectively (Santulli et al., 2008; Yasukawa et al., 2004). Remarkably, enhanced EC monolayer permeability and disrupted VE-cadherin localization at cell junctions were observed in vitro with the α4 integrin antagonist cilengitide (Alghisi et al., 2009). Interestingly, αβ3, a known receptor of Ang-2, was upregulated in high glucose-treated astrocytes. Ang-2-induced astrocyte apoptosis during vascular leakage in STZ-treated animals was attenuated by αβ3 blocking antibodies, suggesting Ang-2/αβ3 integrin signalling as potential therapeutic target during early DR (Yun et al., 2016). Strong anti-leakage effects, comparable to anti-VEGF treatment, were reported for a novel pan RGD integrin antagonist THR-687 in a mouse VEGF-induced permeability model and cynomolgus laser-induced CNV model (Hu et al., 2019) (Fig. 6). THR-687 potently inhibits multiple integrin receptors belonging to the RGD class, including αβ1, αβ3, αβ6, αβ4 and αβ9 with IC50 values in the low nanomolar range (Hu et al., 2019). Retinal edema and VEGF-induced blood vessel permeability were also
suppressed by EGT022, an RGD-containing disintegrin originated from the human-derived protein ‘a disintegrin and metalloproteinase 15’ (ADAM15), in the ROP model and in a modified Mile’s assay, respectively (Jang et al., 2016).

Such findings reinforce the concept that RGD integrin antagonism, especially for the integrins αvβ3 and αvβ5, offers promising options for preventing vaso-permeability occurring in retinal vascular disorders. In addition, these studies underpin the integrated nature of RGD-binding integrins in multiple signalling cascades and their interaction with several vascular leakage-associated growth factors including, but not limited to, VEGF, FGF, Ang-2 and ANGPTL4 (see also section 4).

5.3. Angiogenesis

As already highlighted in section 4, RGD-binding integrins strongly interact with pro-angiogenic growth factors. This dynamic and highly regulated process of new blood vessel growth consists of ECM remodelling, endothelial cell migration, proliferation, survival and adhesion, sprouting and tube formation. The involvement of RGD-binding integrins in each of these steps will be discussed below and is schematically illustrated in Fig. 7. In relation to the current evidence of RGD-binding integrin receptors as therapeutic targets, there is an extensive literature based on animal models of pathological retinal and choroidal angiogenesis.

In the patient context, it is important to note that integrins αvβ3, αvβ5 and αvβ1 have been observed in surgically removed patient CNV membranes. The integrins αvβ3 and αvβ5 were found especially in the early, active stages of disease progression and colocalized with ECs, while αvβ3 expression occurred in the mid and late (fibrotic) stages without colocalization with ECs (Cui et al., 2009). Similarly, Friedlander et al. (1996), observed αvβ3 (not αvβ5) on blood vessels in choroidal neovascular membranes from AMD patients, while both αvβ3 and αvβ5 were present in retinal neovascular membranes from patients with active vasoproliferative retinopathy (Friedlander et al., 1996). These findings indicate different pathological pathways driving retinal and choroidal neovascularization and a distinct role of RGD-binding integrins in these processes (Das and McGuire, 2003).

5.3.1. Extracellular matrix remodelling

For angiogenesis to occur, ECs must invade the surrounding ECM, which can be remodelled by many processes, including synthesis, contraction and proteolytic degradation (Larsen et al., 2006). Proteolytic degradation is mediated by enzymes such as MMPs and plasminogen activators (Theocharis et al., 2016). In ECs, a functional cooperation was observed between MT1-MMP and p1αvβ5 integrins during migration (Galvez et al., 2002). Interestingly, preactivated MMP-2 selectively bound αvβ3, but not αvβ5 integrin, in bovine microvascular ECs and this interaction was inhibited by the cyclic RGD peptide EMD 66203 (Nisato et al., 2005). Blockage of the αvβ3 interaction with MMP-2 via TSR1265 almost completely abolished bFGF-induced angiogenesis in the chick CAM assay (Silletti et al., 2001). Furthermore, the αvβ3 antagonist ATN-161 strongly reduced ocular neovascularization, partially by decreasing expression of MMP-2 and MMP-9, in ROP and laser-induced CNV mouse models (Sui et al., 2018b). More information on the link between integrins and MMPs can be found in section 4.2.3.

A close interaction between integrins and the urokinase-type plasminogen activator (uPA) system has also been described during angiogenesis. The latter system includes, amongst others, uPA and glycolipid-anchored uPA receptor (uPAR) (Tang and Wei, 2008). uPA accumulation was reduced in EC culture medium after exposure to αvβ3 or αvβ5 antagonists (Laurens et al., 2009). In HUVECs, uPAR co-clustered with αvβ1 and knockdown of uPAR or inhibition of the uPAR-integrin interaction by a blocking peptide, prevented VEGF-induced internalization of αvβ1, which is crucial to support angiogenesis (Alexander et al., 2012; Uhrin and Breuss, 2013). Bifulco et al. (2013) described another uPAR blocking peptide that prevented the recruitment of αvβ3 integrin at focal adhesions in VEGF-stimulated ECs (Bifulco et al., 2013). In VEGF-stimulated human retinal ECs, inhibition of uPAR resulted in decreased activation of ERK, p38, JNK and AKT, which is proposed to be
an effect of blocked complex formation of uPAR and αβ2/αβ3 integrin (Motta et al., 2011). Thus, there is evidence to support a role for RGD-binding integrins in ECM remodelling by ECs via MMPs and the uPA system.

5.3.2. Endothelial cell migration
ECs undergoing angiogenic processes express αβ3, αβ5 and αβ1 integrins (see also section 3.4) (Friedlander et al., 1996; Brooks et al., 1994; Tiwari et al., 2011). Treatment of HUVECs with a pan-RGD-binding integrin antagonist (THR-687), an RGD-containing peptide disintegrin derived from snake venom (DisBa-D1) or a non-peptide αβ3 antagonist (GOPPP, 3-(3-guanidino-1-o xoisoindolin-2-yl) propanamido)-3-(pyrindin-3-yl) propanoic acid dihydrochloride), resulted in decreased EC migration (Danilucci et al., 2011; Hu et al., 2015; Li et al., 2014). Furthermore, endostatin, an anti-angiogenic derivative of type XVII collagen, can compete with RGD cyclic peptide to bind integrin αβ3β3, thereby inhibiting EC migration without affecting proliferation (Sudhakar et al., 2003). Studies have shown that ligand binding to one integrin can inhibit the function of other integrins, a phenomenon called integrin cross-talk. For example, in HUVECs, antagonists of integrin αβ3 integrin suppressed αβ3-mediated focal contact formation and cell migration on vitronectin without inhibiting cellular attachment (Kim et al., 2000).

It was described that the chemokine (C-X-C motif) ligand (CXCL)-4 function as a ligand for RGD-binding integrins, such as αβ2, αβ3 and αβ1. Indeed, it was reported that HUVECs adhere to immobilized CXCL-4 through different RGD-binding integrins, supporting EC spreading and migration in an integrin-dependent manner. Soluble CXCL-4 on the other hand inhibited integrin-dependent EC adhesion and migration (Ailoudi et al., 2008), contributing to its anti-angiogenic effect.

5.3.3. Endothelial cell proliferation and survival
EC proliferation is tightly regulated by cell-ECM and cell-cell adhesion, as well as growth factors, cytokines and hormones. Of these environmental cues, the integrins are the most critical as they can regulate mammalian cell cycle progression through FAK and G1 phase cyclin-dependent kinases (CDKs). Integrin signalling has been associated with the induction of cyclin D1 and the downregulation of CDK inhibitors, thereby stimulating cell proliferation. In brief, integrins can increase cyclin D1 expression through ERK and PI3K activation, stimulate translation of cyclin D1 mRNA through Rac and stabilize cyclin D1 through PI3K (Moreno-Layseca and Streuli, 2014; Schwartz and Assoian, 2001; Zhao et al., 1998). In addition, FAK participates in multiple growth regulatory events such as JNK-mediated transcription of insulin.
RGD-binding integrins have also been implicated in EC survival, although the cell signalling pathways are complex. For example, EC adhesion mediated by $\beta_1$ or $\alpha_v$-integrins induced tyrosine phosphorylation and activation of the EGF receptor, and led to MAPK/ERK activation and EC survival (Moro et al., 1998; Perruzzi et al., 2003). Furthermore, the ligation state of $\alpha_v\beta_3$ integrin directly influenced p53 activity and bax-triggered cell death pathways. Whereas antagonists for $\alpha_v\beta_3$ integrin caused activation of p53, agonists suppressed this activity (Stromblad et al., 1996). Furthermore, the RGD-binding integrin inhibitor BCH-15046 was shown to induce apoptosis of ECs plated on vitronectin or type I collagen (Meerovitch et al., 2003). Moreover, the cytoplasmic domain of unligated $\beta_3$ integrin interacted with and activated caspase-8 to induce EC death (Stupack et al., 2001). In fact, the anti-angiogenic activity of the $\alpha_v$ integrin antagonists clengitide, AV-38/398 and S 36578-2 was attributed to anokis, i.e. cell death mediated by cell detachment, through activation of caspase-8 and -9 (Christensen et al., 2016; Maubant et al., 2006). Furthermore, the RGD integrin inhibitor BCH-15046 was shown to induce apoptosis of ECs plated on vitronectin or type I collagen (Meerovitch et al., 2003). Additionally, $\alpha_v\beta_3$ integrin binding of osteopontin and vitronectin stimulated EC survival through NF-κB activation via Src and the small GTP-binding protein Ras (Scatena et al., 1998). Correspondingly, besides inhibiting MMP expression, the $\alpha_v$ integrin inhibitor ATN-161 was able to reduce retinal and choroidal neovascularization in ROP and laser-induced CNV mouse models, respectively, by decreasing NF-κB activation and promoting vascular EC apoptosis (Sui et al., 2018b). Integrin $\alpha_v\beta_3$ also suppressed protein kinase A activity, which induced apoptosis via caspase-8 activation (Kim et al., 2002). The integrin $\alpha_v\beta_3$ antagonist JSM6427 induced apoptosis of cultured ECs, probably by blocking fibronectin-$\alpha_v\beta_3$-mediated cell survival pathways as JSM6427 reduced fibronectin-induced phosphorylation of ERK (Umeda et al., 2006).

5.3.4. Endothelial cell sprouting, tube formation and vessel maturation

In the postnatal mouse retina, angiogenic blood vessels grow and sprout along a pre-formed latticework of astrocytes that express and assemble fibronectin (Kubota and Suda, 2009; Stenzel et al., 2011). Conditional deletion of integrin $\alpha_v$ or $\beta_3$ in astrocytes or integrin $\beta_1$ in ECs led to impaired vascularization of the retina (Hirota et al., 2011; Yamamoto et al., 2015). Several RGD integrin antagonists have demonstrated potent anti-angiogenic effects in a bFGF-driven (SF0166 or JSM6427) or TAT PTD-endostatin-RGD model. Integrin $\alpha_v\beta_3$ inhibition with SF0166 or JSM6427 reduced vascularization and vessel maturation, as shown by treatment with anti-$\alpha_v$ integrin antibodies (Scheppke et al., 2012). RGD-binding integrins also modulate the Ang/Tie signalling pathway, which plays an essential role in vascular stability. This is described in more detail in section 4.1.2.

Taken together, evidence indicates that RGD-binding integrins are essential for several angiogenesis-related processes, including EC proliferation, migration, sprouting and maturation, indicating RGD-binding integrin antagonism as a promising treatment strategy for pathological angiogenesis.

5.3.5. Integrin antagonists in animal models of retinal and choroidal neovascularization

Previous studies have demonstrated the involvement of RGD-binding integrins in the regulation of EC proliferation, survival and migration during angiogenesis. These integrins include $\alpha_v\beta_3$, $\alpha_6\beta_1$, $\alpha_6\beta_3$ and $\alpha_5\beta_1$ (Avraamides et al., 2008; Yue et al., 2012). Systemic or local administration of integrin $\alpha_v\beta_3$, $\alpha_6\beta_3$ and $\alpha_{5}\beta_1$ antagonists can inhibit retinal and choroidal angiogenesis in animal models of ocular disease, such as the collagen-induced vascular disease model (CIVD), indomethacin-induced choroidal neovascularization of SF166 or TAT PTD-endostatin-RGD, which specifically binds $\alpha_v\beta_3$ significantly reduced pathological neovascularization in the ROP model (Askew et al., 2018; Li et al., 2016). The $\alpha_v$ integrin antagonist JNJ-26076713 also dose-dependently inhibited retinal neovascularization in the ROP model after oral administration (Santulli et al., 2008). A reduction in VEGF and VEGFR2 levels, as well as in pathological retinal neovascularization, was observed after intraarterial treatment of the $\alpha_v\beta_3$ and $\alpha_5\beta_3$ integrin antagonist SB-267268 in the same model.
Subcutaneous injection of a cyclic α5-integrin antagonist or the RGD-containing disintegrin EGT022 was also able to prevent hypoxia-induced retinal neovascularization in the mouse eye (Hammes et al., 1996) or increase pericyte coverage and thereby stimulating retinal microvesSEL maturation (Jang et al., 2016), respectively. By blocking the interaction of αβ1 with fibronectin, sustained subcutaneous administration of JSM6427 did not only suppress laser-induced CNV, but could also induced regression of previously established CNV in mice (Umeda et al., 2006).

Beside these studies, showing an anti-angiogenic efficacy via topical, oral, intraperitoneal or subcutaneous delivery, several others confirmed this effect after IVT administration of RGD-binding integrin antagonists in animal models of retinal or choroidal neovascularization. By interacting with αβ1 and α-containing integrins in an RGD-independent manner, laser-induced CNV prevention and regression were obtained after single IVT administration of lebecetin in mouse eyes (Montassar et al., 2017). This heterodimeric C-type lectin also demonstrated inhibition of retinal neovascularization in the mouse ROP model (Montassar et al., 2017). Single IVT delivery of the αβ3 and αβ6 integrin antagonist peptide C16Y significantly inhibited laser-induced CNV, which was even more pronounced using a C16Y nanoparticle solution (Kim and Csaky, 2010). OCU200 (Octogen), a tumstatin – transferrin fusion protein, inhibited as well new blood vessel formation after IVT administration in the ROP and laser-induced CNV model by binding to αβ3 integrins. IVT treatment with the αβ3 RGD integrin antagonist JSM6427, or with the αβ3 integrin antagonists tetrac and GOPPP, also reduced the number of preretal nuclei or neovascular tufts in the ROP model (Li et al., 2014; Maier et al., 2007; Santulli et al., 2008; Yoshida et al., 2012). While the avascular area was increased after JSM6427 treatment or remained unaltered after local tetrac administration, vaso-oblitiration was significantly decreased by GOPPP, which indicates promotion of normal vessel growth. In addition, GOPPP treatment significantly inhibited ERK1/2 phosphorylation and hypoxia-inducible factor 1α (HIF-1α) and VEGF levels in the ROP model. Rat eyes treated IVT with the α1 integrin antagonist cyclic RGD peptide showed significant inhibition of laser-induced CNV and IVT administration of the specific αβ1 inhibitor ATN-161 was described to reduce SDF-1-mediated CNV and leakage in the laser model (Lyu et al., 2018; Sui et al., 2018b; Yasukawa et al., 2004). Moreover, combination therapy with ATN-161 and an anti-VEGF antibody in a rat CNV model showed a stronger anti-angiogenic effect as compared to either agent alone (Wang et al., 2016). Analogously, combined IVT treatment with the αβ3 and αβ1 inhibitor AXT107 and affiberecept, a soluble decoy receptor that inhibits both VEGF and PlGF, in a mouse CNV experiment exhibited a synergistic, suppressive effect on subretinal neovascularization (Silva et al., 2017). When AXT107 was applied in the mouse ROP model, it significantly suppressed ischemia-induced retinal neovascularization (Silva et al., 2017). Also ALG-1001 demonstrated a strong reduction in laser-induced CNV by more than 40% and in retinal neovascularization in the mouse ROP model by more than 50%. In addition, combined administration of ALG-1001 and ranibizumab, a recombinant humanized IgG1 monoclonal antibody fragment that binds and inhibits VEGF, resulted in a 35% better performance than either drug alone in reducing neovascularization in a VEGF transgenic mouse model (Boyer, 2012). As such, a substantial number of preclinical observations demonstrate potent anti-angiogenic and normal vessel stabilizing effects of anti-RGD integrin therapy in animal models of ocular disease. These observations are summarized in Table 2. Interestingly, studies that applied a combination therapy with an anti-VEGF agent demonstrated a stronger reduction of pathological neovascularization than a single treatment.

5.4. Fibrosis

Fibrosis is the result of a wound healing response that follows an acute or chronic injury. It involves excessive cell proliferation, migration and ECM deposition/remodelling and adhesion (Roy et al., 2016). In the retina, fibrotic scarring can compromise vision by causing biological damage and mechanical disruption to the visual axis. This is exemplified by epiretinal fibrotic tissue in DR, subretinal fibrosis in nvAMD and tractional fibrotic membranes in proliferative vitreoretinopathy (Friedlander, 2007).

RGD-binding integrins have been implicated in fibrosis, and modulation of integrins has demonstrated profound effects on fibrosis in multiple organs and disease states (Conroy et al., 2016). For example, β3 knockout mice showed a delayed onset of fibrosis after corneal incision (Wu et al., 2019) and αβ3 and αβ1 integrins were present in human proliferative vitreoretinopathy tissue (Guenther et al., 2019; Robbins et al., 1994; Zahn et al., 2010). As previously mentioned, the observed αβ3 integrin immunolabeling on surgically removed human CNV membranes of mid and late stages indicate its involvement during active remodelling and fibrosis, which was much less evident for αβ1 and α5 (Cui et al., 2009). The role of RGD-binding integrins in the different processes and their interaction with growth factors associated with fibrosis is discussed in more detail.

5.4.1. Myofibroblast differentiation

The involvement of fibroblasts in fibrotic processes throughout the body is well established. TGF-β is upregulated and activated in fibrotic diseases and is a crucial regulator in the differentiation of myofibroblasts, the key effector cells in fibrotic diseases. TGF-β-stimulated fibroblasts undergo a reorganization of the actin cytoskeleton and upregulation of α smooth muscle actin (αSMA) expression, which is often used as a myofibroblast marker (Biernacka et al., 2011; Evans et al., 2003). However, the retina is devoid of fibroblasts, so other cell types including glial cells, RPE cells, ECs and macrophages may contribute to retinal fibrosis, as their presence in epiretinal membranes has been demonstrated (Bringmann et al., 2009; Ishikawa et al., 2016; Little et al., 2018; Tamiya et al., 2010; Tsotridou et al., 2020). In brief, damaged RPE cells that have lost cell-cell contact have been reported to undergo epithelial-to-mesenchymal transition (EMT) and contribute to fibrotic processes in the retina (Tamiya et al., 2010), whereas ECs in DR and nvAMD may transdifferenate into myofibroblasts through endothelial-to-mesenchymal transition (EndoMT) (Cao et al., 2014; Sun et al., 2018). Both myofibroblast differentiation via EMT and EndoMT is mediated by TGF-β signalling. The cross-talk between α integrins and TGF-β signalling is extensively described in section 4.1.3. Additionally, there is substantial evidence for RGD-binding integrins during EMT and EndoMT. Likewise, EMT in bronchial epithelial cells was mediated by integrin αβ3 (Liu et al., 2020). Furthermore, overexpression of integrin β3 promoted EndoMT (Wang et al., 2018), and knock-down of integrin β1 suppressed EndoMT in ECs (Shi et al., 2015).

5.4.2. Extracellular matrix deposition and contraction

Fibroblasts respond to injury by synthesizing various ECM components and thereby mediating a reparative process (Evans et al., 2003; Roy et al., 2016). In the eye, multiple (transdiffereniated) cell types contribute to this ECM deposition, such as ECs, RPE cells and macroglia (Miller et al., 2017). Altered ECM composition is not only a consequence but also a driver of fibrosis. An early step in pathological ECM accumulation is fibroblastic matrix assembly, indicating that RGD-binding integrin-directed therapies likely impact fibrotic diseases by interfering with cell-fibrogenic interactions and as such with matrix deposition (Miller et al., 2017). Importantly, a key role for fibrogenic EMT in complications characteristic of proliferative vitreoretinopathy, including retinal detachment, was demonstrated in rodent models of retinal injury. Administration of nonpeptidic RGD mimetics SF-6.5 and NS-11 inhibited the adhesion of human tenon’s capsule fibroblasts to fibronectin in culture (Hershkoviz et al., 1994). In respect of the eye, αβ1 inhibition by JSM6427 was found to concentration-dependently block attachment of PMA-activated ARPE-19 cells to fibronectin, and to inhibit bFGF-stimulated migration of ARPE-19 cells towards
fibronecin (Zahn et al., 2010). ARPE-19 adhesion to fibronecin could not be inhibited by α5β3 or α1β5 blocking antibodies (Zahn et al., 2010).

ECM stiffness is sensed via inside-out and outside-in signalling across cell adhesions composed of integrins and focal adhesion complexes. Increased matrix stiffness is coupled to elevated levels of several integrins such as integrin β1, αβ2 and α5β1 (Balcioglu et al., 2015; Dong et al., 2019; Yeh et al., 2017). Furthermore, in response to elevated matrix stiffness, cell contractile forces increase and thereby induce a conformational change in the LAP-TGF-β latency complex, resulting in the release of TGF-β and activation of a positive feedback loop of ECM synthesis and stiffening (Wipff et al., 2007). This feedback loop was mediated by TGF-β-SMAD signalling, which induces collagen and fibronecin gene expression (Biernacka et al., 2011) and inhibits MMP-dependent matrix degradation (Edwards et al., 1987). Wipff et al. (2007) indicated that integrin-mediated myofibroblast contraction activates TGF-β3, as both RGD peptides and an integrin α5β3 blocking antibody inhibited latent TGF-β activation. Moreover, treatment of lung fibroblasts with the pan-α5 blocking antibody abituzumab reduced αSMA expression. IL-6 production and collagen gel contraction (Samy et al., 2017). Additionally, two disintegrins, echistatin and flavodin, were able to inhibit RPE cell-induced vitreous contraction and tractional retinal detachment in vitro and in the rabbit eye, respectively (Yang et al., 1996). Lygoe et al. (2004) demonstrated that function blocking antibodies against α5 or β1 could suppress TGF-β-induced αSMA expression in three human fibroblast cell lines. Furthermore, antibodies against α1, α5β1, or α5β3 could inhibit fibroblast contraction in a collagen gel contraction assay, without affecting adhesion.

These findings are in agreement with the observed anti-fibrotic properties of the pan-RGD-binding integrin antagonist THR-687 (see Fig. 8). THR-687 significantly reduced collagen contraction induced by both human dermal fibroblasts (Fig. 8a and b) and ARPE19 cells in a concentration-dependent manner (Fig. 8d and e). Furthermore, THR-687 significantly decreased the expression of αSMA in fibroblasts (52% reduction at 20 μM) and ARPE19 cells (38% reduction at 10 μM) compared to vehicle-treated cells (Fig. 8c, f). Moreover, repeated IVT injections of THR-687 in the cynomolgus laser-induced CNV model dose-dependently inhibited collagen deposition (Sirius Red staining) in the laser spots, although only the highest dose (4.5 mg/eye) induced a significant reduction (Fig. 8g and h).

5.4.3. Gliosis

In the mammalian retina, three main types of glial cells – astrocytes, Müller cells and resident microglia – serve to maintain retinal homeostasis. Reactive gliosis describes the response of astrocytes and Müller cells to injured or diseased tissue in the central nervous system (Fischer and Bongini, 2010). Müller cell gliosis involves cellular hypertrophy, increased proliferation, inflammation as well as tissue and vascular remodelling. Following retinal damage, the expression of vimentin and glial fibrillary acidic protein (GFAP) are dramatically upregulated in Müller cells and astrocytes (Bringmann et al., 2006; Lewis and Fisher, 2003). This increases the stiffness of the cells, thereby discouraging axonal regeneration (Bringmann and Wiedemann, 2012). In severe forms of reactive gliosis, cells can form glial scars that are inhibitory to neuronal regeneration (Sofroniew, 2009).

Robel et al. (2009) demonstrated that β1-integrin-mediated signalling in astrocytes was required to promote their acquisition of a mature, non-reactive state (Robel et al., 2009). Conditional deletion of β1-integrin in Müller cells resulted in partial reactive gliosis including astrocyte hypertrophy and the upregulation of GFAP and vimentin, yet without proliferation (Robel et al., 2009). Similar results were reported with β1-integrin deficient ependymal stem cells, where astrocyte differentiation was suppressed by β1-integrin via the integrin-linked kinase (ILK) pathway (Pan et al., 2014). Moreover, the α5β3 integrin binding agonist, C16, alleviated astrogliosis and demyelination in an acute experimental allergic encephalomyelitis rat model (Han et al., 2013). Contrarily, over-expression of β3-integrin in astrocytes was sufficient to induce astrocyte reactivity (Lagos-Cabre et al., 2017), suggesting a delicate balance between the mature and reactive state of glial cells.

In the eye, IVT treatment with the α5β1 small molecule inhibitor JSM6427 was shown to significantly reduce the number of proliferating Müller cells in a rabbit model of retinal detachment, together with a reduction in the number and length of subretinal scars (Zahn et al., 2010). Postnatal mouse retinas with a conditional deletion of αv or β3-integrin in astrocytes and neurons exhibited reactive gliosis in response to haemorrhage (Hirota et al., 2011).

Overall, RGD-binding integrins and especially αv integrins have been positively linked to fibrotic responses such as myofibroblast differentiation and contraction and could be a valuable target to tackle retinal fibrosis. Although some RGD-binding integrins seem crucial for the mature, non-reactive state of glial cells, validation studies with pharmacological inhibitors are needed to confirm these cell type-specific, integrin knockout findings.

As described in these sections and summarized in Fig. 9, there is a growing amount of preclinical evidence indicating an important role for RGD-binding integrins in the pathogenesis of DR and nvAMD, demonstrating clinical promise with RGD-binding integrin intervention in these vision-threatening disorders (Bhatwadekar et al., 2020).

6. Clinical evidence on the role of RGD-binding integrins in diabetic retinopathy and neovascular-age-related macular degeneration

6.1. Diabetic retinopathy

DR is the most common microvascular disorder caused by diabetes mellitus and a leading cause of blindness in working-aged people in industrialized countries. DR can be broadly classified into a non-proliferative and a proliferative stage. The first discernible symptoms of non-proliferative DR are microaneurysms and retinal haemorrhages. Proliferative DR occurs with further retinal ischaemia and is characterized by the growth of pathological blood vessels on the surface of the retina. These new vessels are unstable and may leak, leading to vitreous haemorrhage. DME is a complication of DR characterized by fluid accumulation in the macula of patients and can occur at any stage of the disease (Ford et al., 2013; Gao et al., 2008; Mohamed et al., 2007). For many years, DR was considered as a vascular eye disorder, but now, it is generally acknowledged that pathological processes such as oxidative stress, neurodegeneration, chronic inflammation, gliosis and fibrosis also play a major role in the pathogenesis of early as well as advanced DR (Brownlee, 2005; Curtis et al., 2009; Stitt et al., 2013, 2016).

The current first-line treatment for centre-involved DME are anti-VEGF agents (i.e. Eylea® or aflibercept, Lucentis® or ranibizumab and the off-label used Avastin® or bevacizumab). Even though anti-VEGF therapy has proven to be effective, various reports have stated that a subset of the DME patients respond sub-optimally to anti-VEGF treatment and display persistent edema with associated visual function loss (Nguyen et al., 2010, 2012). These findings suggest that other pathways, in addition to VEGF, contribute to the development of DME. Alternative therapies of advanced DR consist of IVT corticosteroids injections, retinal laser photocoagulation or surgical vitrectomy. However, all these currently available therapies suffer from potential significant side effects. It has been reported that repeated treatment with VEGF inhibitors can result in increased risk of fibrotic complications and tractional retinal detachment (Moradian et al., 2006) and even signs of neurodegeneration have been reported (Beck et al., 2016). Corticosteroids come with high risk of cataract formation and increased intraocular pressure (Beck et al., 2016; Nuzzozer and Unlu, 2017; Yamamoto et al., 2003). Hence, numerous pharmaceutical companies endeavour to discover novel, alternative drug candidates for DR and DME with a superior long-term effectiveness and safety profile, and the potential to reduce the treatment burden.
Fig. 8. THR-687 can inhibit fibrosis in preclinical models. Human dermal fibroblasts (HDF) or human retinal pigment epithelial cells (ARPE19), seeded in (A–B) or on top (D–E) of collagen gels, respectively, were treated with different concentrations of THR-687. A negative control was taken along to exclude intrinsic contractile properties of the gel (no HDF/no ARPE19). Contraction was monitored during 14 or 4 days for HDF or ARPE19, respectively (A, D) (protocols adapted from (Lygoe et al., 2004; Morales et al., 2007)). Images were quantified by measuring the area of the gel over the area of the well (B, E). Afterwards, cells were extracted from the collagen gels and alpha smooth muscle actin (αSMA) protein levels were measured (n = 3–4 independent experiments) (C, F). Cynomolgus monkeys received 9 laser spots to induce choroidal neovascularization (CNV) and were given either vehicle (3x IVT weekly), Lucentis (0.5mg/eye; 1x IVT) or THR-687 (0.45mg/eye, 2.25mg/eye or 4.5mg/eye; 3x IVT weekly). Animals were sacrificed at day 22 and collagen deposition in the lesion area (red dotted line) was measured by staining ocular paraffin sections with Sirius Red (SR) (G). Quantification was performed by measuring the Sirius Red positive area over the total lesion area (n = 3–6 eyes/condition). (H). Panel A–F represents new data, whereas panel G–H represent a new analysis of samples from a previously published study (Hu et al., 2019).

Data are mean ± SEM and analyzed via one-way ANOVA with post hoc Bonferroni test (GraphPad Software, *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).
There is clinical evidence that integrins play an essential role in the pathogenic processes of DR and DME. Immunostainings on human retinal tissues derived from PDR patients revealed that actively proliferating vascular ECs express increased $\alpha_v\beta_3$ and $\alpha_v\beta_5$ levels (Friedlander et al., 1996). In general, DR has not been found to be strongly correlated with genetic mutations or polymorphisms. Among the candidate genes are aldose reductase, the receptor for advanced glycation end products, with genetic mutations or polymorphisms. Among the candidate genes, $\alpha_3\beta_1$ and $\alpha_5\beta_1$, but also integrins from other classes, namely $\alpha_5\beta_1$ and $\alpha_6\beta_1$ (Shaw et al., 2020; Tolentino et al., 2016). Risuteganib has been evaluated in a randomized, prospective, double-masked phase 2b trial for DME patients (NCT02348918, DEL MAR study) where its safety and effectiveness were compared to bevacizumab. In stage 1 of the DEL MAR monotherapy study, 136 subjects were randomized to three risuteganib groups (1.0, 2.0, or 3.0 mg) treated with 3 monthly IVT injections, followed by 3 months off-treatment, and a 1.25 mg bevacizumab arm with 6 monthly IVT injections. This trial met its primary and secondary endpoints of non-inferiority in best corrected visual acuity (BCVA) improvement as well as reduction in central macular thickness (CMT) as compared to bevacizumab. In the stage 2 of the DEL MAR study, 80 participants with DME were randomly assigned to 1 of 5 treatment groups. The control group received monotherapy of five monthly injections of 1.25 mg bevacizumab, while two other groups received sequential therapy of a single IVT injection of 1.25 mg bevacizumab at week 0 followed by three IVT injections with 1.0 or 0.5 mg risuteganib at weeks 1, 4 and 8. The two last groups received combination therapy of 1.25 mg bevacizumab with 1.0 or 0.5 mg risuteganib IVT injected at weeks 1, 4 and 8. The sequential therapy regimen with 1 mg risuteganib was most efficacious and met the primary endpoint of non-inferiority in BCVA gain when

Fig. 9. Overview of the most relevant RGD-binding integrins contributing to the pathological processes implicated in retinal vascular disorders.

| Drug code or INN     | Company                      | RGD integrin receptor subtype target | Indication | Trial      | Status     | NCT number          | References                                      |
|----------------------|------------------------------|-------------------------------------|------------|------------|------------|---------------------|------------------------------------------------|
| Risuteganib          | Allegro Ophthalmics          | $\alpha_5\beta_2$, $\alpha_3\beta_1$, $\alpha_2\beta_3$, $\alpha_5\beta_3$ | nvAMD      | Ph1/2      | Completed  | NCT01749891        | (Quirós-Mercado, 2012; Shaw et al., 2020)       |
| THR-687              | Ocuron                       | $\alpha_5\beta_2$, $\alpha_3\beta_1$ | DME        | Ph2        | Completed  | NCT02348918        | (Dugel, 2019; Shaw et al., 2020)                |
| AXT107               | AsclepiX Therapeutics        | $\alpha_5\beta_3$, $\alpha_3\beta_1$ | DME        | Ph1/2a     | Planned    | NCT03666923        | Ocuron (2020)                                   |
| OTT-166              | OcuTerra Therapeutics        | $\alpha_5\beta_3$, $\alpha_5\beta_1$, $\alpha_1\beta_3$ | nvAMD      | Ph1/2a     | Ongoing    | NCT04697758        | (AsclepiX Therapeutics)                         |
| ASI101               | Feramda Biopharmaceuticals   | $\alpha_5\beta_3$                  | DME        | Ph1/2      | Completed  | NCT02914639        | (Askew et al., 2018; Dugel, 2019; Eyewire.news, 2017) |
| JSM-6427             | Jerini AG & Shire Pharmaceuticals | $\alpha_5\beta_1$                 | nvAMD      | Ph1        | Completed; no further studies planned | NCT00536016 | (Capone et al., 2009) |

CNV: choroidal neovascularization; DME: diabetic macular edema; INN, international non-proprietary name; nvAMD: neovascular age-related macular degeneration.
compared to bevacizumab, with fewer total injections. A 12-week durability after the last dose of risuteganib was demonstrated with sequential therapy. Remarkably, combination therapy was far inferior to sequential therapy as well as bevacizumab monotherapy. Of note, patients who had been sub-optimal responders to prior anti-VEGF therapy appeared to have a better response to risuteganib treatment (Dugel, 2019; Shaw et al., 2020). Additional (clinical) research is needed to better understand how anti-integrin therapy could fit into the current treatment regimens for DR and DME.

Second, SF0166 (OcuTerra Therapeutics, formerly SciFluor Life Sciences, Inc.) is a potent \( \alpha_5\beta_3 \) antagonist which was topically administered in a phase \( \frac{1}{2} \) study for DME patients (NCT02914613). In this study, safety and preliminary efficacy of SF0166 were investigated in 40 DME patients who were randomized to a dose of 2.5% or 5% eye drop formulation, self-administered twice-a-day for 28 days. The primary endpoint was reached given that no drug-related serious adverse events were observed throughout the study. Both doses of SF0166 exhibited therapeutic efficacy with 53% of the subjects displaying a decline in retinal thickness, and BCVA improvements were also reported. Durability of retinal thickness response to SF0166 treatment was observed during the 28-day follow-up period after discontinuing treatment (Askew et al., 2018; Dugel, 2019). Although the data from the phase \( \frac{1}{2} \) study of SF0166 did seem to indicate a biological effect, no further development of this compound has been reported to date. In addition, up to now, no topically administered drug is approved for the treatment of DR or AMD.

Third, given the favourable preclinical safety and efficacy profile for the novel small molecule integrin receptor antagonist THR-687 (Oxu- rion NV) (see section 5) (Hu et al., 2019), a clinical phase 1, open label, multicentre study (NCT03666623) was carried out to investigate the safety of a single IVT of THR-687 using three dose levels (0.3, 0.8 and 2.1 mg per eye, expressed as free base) for the treatment of DME (n = 12). THR-687 was found to be safe and well tolerated at all dose levels. Following a single IVT injection of THR-687, a rapid onset of action in mean BCVA was observed as of day 1 with a 3.1 letter gain. The highest impact was observed one month post injection, showing a mean increase of 9.2 letters. The improved vision was maintained up to month 3, with a mean 8.3 letters improvement. BCVA improvement was most pronounced in the high dose group, with a mean BCVA gain of 12.5 letters at month 3. For the high dose the retinal thickness decreased by a mean of 106 \( \mu \)m at day 14 post administration.

### 6.2. Neovascular age-related macular degeneration

AMD is the leading cause of blindness in elderly people of industrialized countries and can be divided into a wet/neovascular/exudative (nvAMD) subtype (10–20% of the patients) and a dry/atrophic/non-exudative form (80–90%). According to the severity of the fundus lesions, AMD is currently classified into early, intermediate and advanced stages. Visual acuity is generally unaffected in the early and interme- diate stages of the disease, which are primarily characterized by the formation of drusen or pigmented abnormalities between RPE cells and Bruch’s membrane. In the advanced stages, AMD might progress into the currently untreatable geographic atrophy, which is characterized by areas of progressive RPE cell degeneration, subsequent photoreceptor damage and finally irreversible loss of visual function (Bandello et al., 2017; Ferris, III et al., 2013; Gehrs et al., 2006), or into nvAMD, where pathological blood vessels from the choroid invade the macula (Gehrs et al., 2006). CNV is mostly accompanied by subsequent development of subretinal fluid accumulation, haemorrhage, fibrosis and retinal detachment (Little et al., 2018; Ma et al., 2017). Though less prevalent, nvAMD is the leading cause of visual loss as it can lead to legal blindness within six months. Dry AMD is mostly characterized by slow progress- sion, yet, 10–15% of patients with dry AMD progress into nvAMD. AMD is a multifactorial disorder driven by oxidative stress, cell senescence, inflammation, dysregulated lipid metabolism and mitochondrial dysfunction. More information on the mechanisms behind AMD can be found in the reviews of Ambati and Fowler (2012) and Rozing et al. (2020) (Ambati and Fowler, 2012; Rozing et al., 2020). A combination of environmental risk factors such as smoking, hypertension, atherosclerosis, high cholesterol and UV light (Cougnard-Gregoire et al., 2013; Dasari et al., 2011; Garcia-Layana et al., 2017; Lim et al., 2012; Thornton et al., 2005), as well as genetic variants in e.g. complement factors (CFH, CFB, C3), Apolipoprotein E, and HTRA1 (Dewan et al., 2006; McKay et al., 2011; Tzoumas et al., 2021; Yang et al., 2006) contribute to AMD pathology. Although there is currently no treatment for dry AMD patients, prevention of AMD onset is mostly based on supporting/maintaining a healthy lifestyle with regular exercise, no smoking and a nutritious diet rich in green, leafy vegetables. Dietary supplements such as the AREDS2 can slow the progression towards the advanced stages of the disease (Agron et al., 2021).

The ground-breaking introduction of IVT anti-VEGF therapy, such as ranibizumab, aflibercept and bevacizumab, has significantly reduced the incidence of blindness in nvAMD patients. However, the functional outcome following anti-VEGF therapies in people with this advanced form of neovascular AMD is in most cases limited (Ishikawa et al., 2015). Indeed, in comparison to clinical trials, patients are usually undertreated in real-world practice, thereby leading to a decline in visual acuity over time, which might be caused by fluctuations in CST (Avery et al., 2020). Besides encouraging patient compliance and improving patient monitoring, more durable anti-VEGF agents and alternative therapies should be developed. Long-term anti-VEGF treatment in elderly people with nvAMD has also been correlated to potential adverse events, namely enhanced scar formation (Daniel et al., 2014), RGC damage (Beck et al., 2016) and potential development of macular atrophy (Gemenetzti et al., 2017).

Interestingly, it has been reported that high levels of \( \alpha_5\beta_3 \) integrin were observed in human neovascular choroidal membranes (Friedlander et al., 1996) and recent clinical trials with compounds targeting RGD-binding integrins have shown promising results for nvAMD patients (see also Table 3). Besides DME, nvAMD was also investigated as an ocular indication for risuteganib (Allegro Ophthalmics) (see also section 6.1). In a phase 1b study, 15 subjects with nvAMD received three monthly IVT injections of risuteganib, which was well tolerated and resulted in a mean improvement in BCVA of five letters as well as a 30% decline in central macular thickness (Shaw et al., 2020). Analogously, a double masked phase \( \frac{1}{2} \) study (NCT02914639) evaluated the safety and preliminary efficacy of SF0166 (OcuTerra Therapeutics, formerly Sci- Fluor Life Sciences, Inc. see also section 6.1) in 42 nvAMD patients, who were randomized 1:1 to self-administer a topical formulation of 2.5% or 5% SF0166 twice-a-day for 28 days (Eyewire, 2017). The primary endpoint was reached since no drug-related serious adverse events were observed throughout the treatment period of 28 days as well as the 28-day follow-up period. In addition, topical SF0166 treatment led to a clinically significant therapeutic effect in nine out of the 42 patients. A mean improvement in BCVA of approximately five letters and a decrease in central retinal thickness and/or subretinal fluid was observed. To our knowledge, no further development in nvAMD was currently reported for both compounds. Besides these, additional phase 1, open label, dose escalation studies were performed with intravitreal \( \alpha_5\beta_1 \) inhibitors, i.e. volociximab (Ophthotech, currently IVERIC bio, NCT00782093) and JSM6427 (Jerini Ophthalmic, currently Takeda Pharmaceutical Company, NCT00536016). Despite the preliminary favourable safety profiles and the promising results (JSM6427, NCT00536016), no further development of these compounds for nvAMD has been reported to date. A phase \( \frac{1}{2} \) clinical trial also recently investigated the safety and efficacy of AS101 (1% oral solution) in patients with nvAMD, but no results are available at the moment (Feranda Biopharmaceutical, NCT03216538). Although AS101 has a wide activity profile, this oral drug is also a functional inhibitor of specific integrins, including \( \alpha_5\beta_3 \), \( \alpha_4\beta_1 \) and the RGD-binding integrin \( \alpha_6\beta_3 \) (Dardik et al., 2016; Lee et al., 2014c; Yossipof et al., 2019).
7. Future directions and conclusions

Recent clinical trials for novel drug candidates developed against RGD-binding integrins have shown promising effectiveness and a favourable safety profile in patients with DME and nvAMD. This has triggered renewed interest in ophthalmic therapies targeting RGD-binding integrins and new inhibitors which have been generated in the last few years. Indeed, while risuteganib, SFO166, and THR-687 already demonstrated biological effects in early clinical trials, promising preclinical efficacy studies with several other anti-RGD integrin drugs including, but not limited to, AXT107 (AsclepiX Therapeutics) and OCU200 (Ocugen) (GlaxoSmithKline), offer further exciting new opportunities. Taken together, there is compelling evidence that RGD-binding integrins are important players in key disease hallmarks of nvAMD and DR, namely chronic inflammation (or para-inflammation), retinal permeability, neovascularization and fibrosis. Moreover, as discussed in this review, RGD-binding integrins, which are present throughout the retina, have the capacity to regulate these vision-threatening processes at multiple levels. They might do so by interacting with various growth factors, cytokines or other stress mediators (e.g. VEGF, Ang-2, TGF-β, bFGF, IL-1β and TNF-α).

Although their specific role in the retina remains incompletely defined, RGD-binding integrins can be considered as functional hubs during pathological signalling and their antagonism could have therapeutic utility for major retinal diseases. Additionally, an RGD-binding integrin inhibitor could have the capacity to act upstream as well as downstream of the VEGF pathway and address VEGF-dependent and VEGF-independent vision-threatening pathological mechanisms, thereby in all likelihood exerting a broader biological effect as compared to anti-VEGF therapy. Moreover, alternative administration routes, such as oral or topical, or optimized dosage forms, such as sustained release formulations, have demonstrated promise in preclinical and/or clinical studies. RGD-binding integrin inhibitors can thus potentially address the treatment burden associated with frequent anti-VEGF injections.

Although RGD-binding integrin inhibitors can have the potential to treat multifactorial and complex eye diseases, possible pitfalls of anti-integrin based therapeutic targets should be taken into consideration since integrins have a wide-ranging impact on many biological functions and fundamental cell signalling cascades. Disruption of normal αβ function can have negative outcomes as demonstrated by β5-integrin knock-out mice showing dysregulated photoreceptor outer segment phagocytosis by RPE cells and impaired vision after 1-year (Nandrot et al., 2004; Nandrot and Finnemann, 2006). In addition, in a rat model of retinal ischemia reperfusion injury, it was described that a decrease in β1-integrin was associated with RGC death via loss of homeostatic RGC-laminin interaction, implying that laminin-binding integrins are essential for RGC survival (Santos et al., 2012). Therefore, as for any new drug candidate, it is of utmost importance to monitor the possible adverse events of anti-integrin therapeutics and to identify a safe therapeutic window. Encouragingly, the abovementioned RGD-binding integrin inhibiting drug candidates all have shown a favourable safety profile in clinical trials. Nevertheless, given the limited number of subjects and relatively short-term study periods, further assessment is needed.

In summary, an RGD-binding integrin inhibitor can be considered as an integrative therapy given its multi-mechanism targeting properties, which is unique amongst all drug candidates being studied for AMD and DR. As integrin functions are complex and context-dependent, further research is recommended in order to unravel the underlying cellular and molecular mechanisms of action when targeting RGD-binding integrins in the context of DME as well as nvAMD. Importantly, it still also needs to be determined if anti-RGD-binding integrin therapy could be non-inferior or superior to anti-VEGF therapy, the current gold-standard of care for DME and nvAMD patients. This would be essential to justify its implementation as a stand-alone therapy and potential first-line treatment. Given the promising results with the sequential therapy regimen in the DEL MAR phase 2b stage 2 clinical study, anti-integrins might also be explored as complementary drugs to anti-VEGF therapy. In addition, it could be useful to evaluate in future clinical studies whether the combination therapy of VEGF and RGD-binding integrin inhibitors could have potential advantages over monotherapy since a synergistic effect has been described before for these two pathways (Silva et al., 2017; Wang et al., 2016). However, as described in section 6.1, a small phase 2b clinical trial in DME patients that received risuteganib and bevacizumab combination therapy was found inferior to bevacizumab monotherapy.

We conclude that it is imperative to continue the search for new and effective drug candidates that could successfully treat all subjects suffering from a complex vision-threatening retinal disorder and we suggest that targeting RGD-binding integrins could be such a next-generation therapy which could revolutionize the treatment paradigm for major sight-threatening and life-altering retinal diseases.

Declaration of interest

Inge Van Hove, Tjing-Tjing Hu, Karen Beets, Tine Van Bergen, Isabella Etienne, Elke Vermassen and Jean H.M. Feyen report direct financial relationship for Oxurion NV. Alan W. Stitt receives remuneration from Oxurion NV for scientific advice.

CRediT authorship contribution statement

Inge Van Hove, Tjing-Tjing Hu, Karen Beets, Tine Van Bergen, Isabella Etienne: Conceptualization, Data curation, Methodology, Investigation, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing; Alan W. Stitt, Elke Vermassen, Jean H.M. Feyen: Supervision, Project administration, Writing – review & editing

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

The authors wish to thank Bernard Noppen, Kelly Vanhulst, Huberte Moreau, Valerie Vanheukelom and Astrid De Vriese for excellent technical support. The graphical abstract and Fig. 3 were created with BioRender.com.

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