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Combined inhibition of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) for neovascular AMD *in vitro*

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Gewidmet meinen Eltern
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is my own unaided work. Moreover, I confirm that no sources other than the ones indicated in the thesis itself have been used. All direct or indirect sources used are acknowledged as references.

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Munich, 28th of May 2019	Jakob Siedlecki
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1 Abbreviations

AMD	age-related macular degeneration
CNV	choroidal neovascularization
CTGF	connective tissue growth factor
DMSO	dimethyl-sulfoxide
EMA	European Medicines Agency
ETDRS	early treatment of diabetic retinopathy study
FDA	US Food and Drug Administration
GA	geographic atrophy
hPC-PL	human pericytes from placenta
HUVEC	human umbilical vein endothelial cells
IC_{50}	half-maximal inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
nAMD	neovascular age-related macular degeneration
OCT	optical coherence tomography
PI3K	phosphoinositide 3-kinase
PCR	polymerase chain reaction
PLC- γ	phospholipase γ
PlGF	placental growth factor
PDGF	platelet derived growth factor
PRN	pro re nata
MAPK	mitogen activated protein kinase
RNA	ribonucleic acid
RPE	retinal pigment epithelium
SMA	smooth muscle actin
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide
VEGF	vascular endothelial growth factor

2 Confirmation of co-authors

Confirmation pursuant to § 4a Paras. 3 and 5 Doctoral Degree Regulations for Dr. med., Dr. med. dent. and Dr. rer. biol. hum. and pursuant to § 7 Para. 4 Doctoral Degree Regulations for Dr. rer. nat. at the Medical Faculty.

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I hereby confirm that the article mentioned above, submitted for this doctoral degree, has not been the subject of another (current or completed) dissertation.

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3 Introduction

The scope of diseases affecting the macula is broad. As this special region provides the highest visual acuity of the retina, it has specific metabolic needs, especially in its central part, the fovea[1]. The retinal pigment epithelium (RPE), which provides metabolic support to the outer retina, has its highest RPE to photoreceptor density subfoveally[2], and also choroidal thickness has been found to be highest in the foveolar region[3-5].

Due to these support mechanisms, aging of the healthy human retina, measured as photoreceptor loss, is paradoxically less pronounced in the fovea and parafoveal area than in the retinal periphery[6,7]. Conversely, in the case of decompensation of these support mechanisms, macular degeneration can cause rapid and severe loss of visual acuity[8].

Age-related macular degeneration (AMD) is the most frequent cause of irreversible visual impairment and blindness in the elderly[9,10], affecting 8.7% of the world population between 45 and 85 years[11]. Due to demographic change, the number of people affected is expected to increase 1.5-fold within 20 years from 2020, equalling 288 million by 2040[11].

Clinically, AMD is defined by the accumulation of Drusen ≥ 63 µm on OCT imaging of the macula[12]. Drusen mainly contain lipid[13] and seem to represent unprocessed debris of the visual cycle[14]. Following Drusen growth and accumulation, AMD progresses via the intermediate into the late stage, which is defined (I) as the ‘wet’ form characterized by the development of pathologic vessels growing from the choroid into the neuroretina, termed choroidal neovascularization (CNV); or (II) the ‘dry’ form, characterized by progressive atrophy of the retinal pigment epithelium, photoreceptors and choriocapillaris, termed geographic atrophy (GA)[8,15].

Visual decline is much more rapid and severe in the ‘wet’ compared to the ‘dry’ phenotype[8,16]. While the wet form makes up 10 to 15 % of all cases of AMD, it is responsible for severe visual disability in about 80 % of AMD patients. This is due to the results of pathologic angiogenesis as represented in CNV: fibrovascular disruption of the neuroretina, leakage of fluid and bleeding[17]. Therefore, management of AMD in its neovascular phenotype mainly represents the management of pathologic angiogenesis, i.e. the re-induction of vascular quiescence, the promotion of regression of pathologic vessels, and the prevention of vessel regrowth[8]. With the advent of Verteporfin photodynamic therapy in 1999, the risk of moderate and severe vision loss

secondary to neovascular AMD (nAMD) could be significantly diminished compared to the natural course, however not offering mean gains in mean visual acuity compared to baseline[18,19]. In 2006, the advent of the first intravitreal anti-VEGF antibody Ranibizumab (Novartis AG, Basel, Switzerland) revolutionized the treatment of nAMD, for the first time offering partial restoration of visual acuity lost due to neovascular disease during a follow-up of two years[20,21]. In the following, non-inferior clinical outcomes were also demonstrated for the antibody Bevacizumab, of which Ranibizumab is a fragment, during a follow-up of 5 years[22].

After a ten-year experience with anti-VEGF, two major limitations have however proven challenging in clinical practice. Firstly, the currently available anti-VEGF substances have to be re-injected frequently due to the chronicity of the disease and several mechanisms of drug clearance from the vitreous body[23-25]. In the pivotal phase three studies, Ranibizumab was therefore injected on a fixed interval of four weeks[20,21], which is difficult to translate into clinical practice due to the burden on patients and health care providers alike, resulting in inconsistent reinjections and frequent discontinuation of therapy in real life[26]. According to current guidelines, in the case of newly diagnosed nAMD, an upload of three injections every four weeks is recommended and has been widely adopted worldwide[8,27]. While there is much consensus on this ‘induction phase’, intensity of treatment in the following ‘maintenance phase’ has been highly debated[28,29]. In the beginning of anti-VEGF therapy, clinicians adopted a pro re nata (PRN) regimen, which, after the upload, moves to monthly monitoring and further treatment in the case of documented loss of visual acuity or OCT showing signs of disease reactivation, defined as re-injection criteria as proposed in the PrONTO study[30]. Experience gained in the following years however showed that (I) reactivation of CNV and recurrence of macular fluid is significantly associated with increased short and long-term loss of visual acuity[31]; and (II) that disease activity and thus intensity of therapy needed are highly individual[30,32]. Therefore, nowadays a ‘treat & extend’ regimen is recommended, which aims at individualizing treatment intervals and reducing control and injection visits while preventing recurrence of edema[28,29]. After an upload of three injections every four weeks, injections are performed at every follow-up visit, but treatment intervals are extended by two weeks in the case of successful disease management, i.e. no signs of macular fluid or CNV reactivation on OCT imaging. In the case of disease reactivation, e.g. recurrence of macular fluid on OCT, the treatment interval is

shortened by two weeks (minimum: 4 weeks). After achieving an interval of 12 weeks, anti-VEGF therapy might be considered to be stopped or, as disease reactivation is frequent, paused[28,29].

Although the treat & extend regimen allows for a reduction of control and injection visits and has been shown to obtain superior results to PRN injections[27], repeated need of diagnostic and therapeutic interventions over many years poses a gigantic socioeconomic burden[33,34]. Further development of anti-VEGF substances has therefore aimed at improved and prolonged anti-neovascular activity; in 2012, bimonthly intravitreal Aflibercept was shown to be non-inferior to monthly Ranibizumab treatment, as second substance gaining approval for the treatment of nAMD[35,36]. However, differences in injection rates and socioeconomic cost between Ranibizumab and Aflibercept are of little clinical significance[34,37], with 4.9 (Ranibizumab) vs. 5.2 (Aflibercept) injections in the first year, as reported in the US[37] or by preliminary data of the RIVAL trial (ongoing).

Secondly, in addition to the high intensity of treatment, mean long-term visual acuity results in nAMD are poor in spite of anti-VEGF therapy. Data from the Seven-Up trial, for the first time offering substantial follow-up beyond two years and indicating a mean loss of 8.2 ETDRS letters after seven years[33], was recently confirmed by the five-year results of the comparison of age-related macular degeneration treatment trials (CATT), which indicate a mean loss of 10.8 letters in comparison to the gains at year two[33]. In addition to this mean loss in visual acuity, about ten percent of patients are classified as anti-VEGF non-responders, losing more than 15 ETDRS letters at two years[20,28,38]. Both the high intensity of treatment, caused by repeated CNV reactivation, as well as the poor long-term outcomes, which are clearly attributed to the presence of intraregional fluid, scarring and atrophy, are signs of not sufficiently managed CNV activity[31]. Therefore, the evaluation of new therapy strategies for nAMD is of great interest, aiming at improving long-term visual outcome, and secondly lowering the treatment burden for patients and health care professionals alike.

In addition to VEGF, a variety of growth factors has been shown to play a major role in nAMD, including platelet derived growth factor (PDGF), placenta growth factor (PlGF) and connective tissue growth factor (CTGF), among others[39]. Of these, the combined inhibition of VEGF and PDGF has been studied most extensively in *in vitro* and *in vivo* oncologic and ophthalmologic studies[40-42]. The interaction between these two growth factors is highly dependent on the vascular status, as quiescent vessels have other

metabolic needs than vessels involved in angiogenesis and vascular remodeling[43]. While physiological vascular quiescence is mediated by simultaneous, concerted VEGF/PDGF action, angiogenesis in health and disease requires sequential growth factor signalling, as it is a result of firstly vessel formation (mediated by endothelial cells and VEGF) and secondly vessel maturation (mediated by cells of the vascular wall, mainly pericytes and PDGF)[43,44].

Vessel formation in CNV is a result of *sprouting angiogenesis,* which can be divided into four steps[45]. Firstly, endothelial cells loosen their intercellular contacts and secrete matrix metalloproteinases, releasing themselves and cells of the vascular wall from the basement membrane[45]. Secondly, endothelial cells migrate into the interstitium[45]. While so-called *tip cells* lead the vascular sprout, their release of VEGF directed backwards causes so-called *stalk cells* to follow[46]. In the third step, the cells proliferate, finally resulting in the formation of tube-like structures, after which the establishment of a new basement membrane follows in the fourth step[45]. All of these mechanisms are mainly VEGF-dependent, and current anti-neovascular therapy is believed to convey its therapeutic properties by the interference with them, reducing vascular leakage and thus retinal edema, inducing partial vessel regression, and delaying CNV regrowth[47].

After these VEGF-driven steps representing vessel formation, PDGF driven vessel maturation establishing stable vascular constructs and endothelial cell VEGF independence is mediated by the recruitment of pericytes and smooth muscle cells[44]. Pericytes mediate this maturation by mechanical stabilization and the paracrine secretion of endothelial cell supporting growth factors, mainly Angiopoietin 1 and VEGF[48]. As pericyte-derived VEGF is secreted in an iuxtacrine/paracrine fashion, common intravitreal anti-VEGF neutralization cannot sufficiently exert inhibitory effects in this case[49,50]. Pericytes themselves are VEGF-independent[44]. Thus, pericyte covered segments of CNV are supposed to be partially anti-VEGF resistant, rendering a substantial percentage of the CNV irresponsible to nowaday's standard of care[51]. This is supported by ongoing OCT angiography studies, as for the first time reported by Huang et al.[47], which show that CNV size can be reduced by anti-VEGF, but CNV cannot be forced into complete regression, and that regrowth from persisting vascular beds re-occurs as anti-VEGF levels drop with time. To start angiogenesis, hypoxic regions of the retina release VEGF, inducing excess formation of new vessels. As reported for the first time by Benjamin et al.[52], pericyte recruitment and association with

newly formed vessels then lag a few days behind. This opens a plasticity window for vascular remodelling, as pericyte-naïve tubes are forced into regression when anti-VEGF levels drop because the newly vascularized retina is not hypoxic anymore[52]. Only larger vascular trunks, having undergone pericyte coverage, then persist in spite of VEGF depletion, and form the new vascular bed[43].

Blockage of the PDGF pathway can disrupt endothelial cell-pericyte communication[40]. In this way, addition of anti-PDGF substances to the arsenal against nAMD might interfere with pericyte recruitment to neovessels, and secondly probably strip pericytes off matured CNV segments. This provides a very interesting new strategy to manage CNV in AMD, as exposing a more vulnerable pericyte-naïve CNV to anti-VEGF would improve neovascular management[44].

The PDGF family consists of five polypeptides of 30 kilodalton (kDa)[53]. Signaling is mediated in a dimerized fashion, resulting in the subforms PDGF-AA, -BB, -CC, -DD and the mixed form -AB[53]. In the eye, signaling is predominantly mediated by PDGF-BB[54-56]. Large global, multicenter, randomized, prospective, double-masked, controlled superiority trials have been designed to study the effect of combined anti-VEGF and anti-PDGF-BB inhibition on nAMD[41,42]. Combined with Ranibizumab or Aflibercept treatment, PDGF inhibition was achieved by the additional intravitreal injection of Pegpleranib (Fovista; Ophtotech, New York USA), an aptamer designed to bind PDGF-BB[41,42]. Published data from the phase IIb trial showed promising results with 62% visual gain in the combination arm (1.5 mg Pegpleranib) compared to Ranibizumab mono, translating into 10.6 vs. 6.5 early treatment of diabetic retinopathy study (ETDRS) letters after 24 weeks[41]. However, the following phase III study failed to confirm superiority[57]. Additionally, the sequential injection of both agents at a volume of 0.05 ml in the same visit, equaling twice the volume of nowadays standard of care, required thorough post-interventional monitoring of intraocular pressure in all study patients and IOP-lowering therapy in some (own experience).

The first anti-VEGF substance to enter the clinic was the aptamer Pegaptanib in 2004 (Macugen; EyeTech Pharmaceuticals, New York, USA)[58]. However, the later antibodies Ranibizumab and Aflibercept have been shown to be of greater potency in suppressing nAMD[59,60]. In a similar fashion, PDGF suppression in pericytes has been shown to be of high anti-neovascular effect *in vitro* and *in vivo*[40,61], but might not be thoroughly addressed by the aptamer Pegpleranib in humans *in vivo*. This is supported by the fact that PDGF, supporting pericyte coverage of neovessels, is secreted as

growth factor in a paracrine fashion and bound to heparan sulfate proteoglycans on the endothelial surface or in the matrix surrounding pericytes, thus being less exposed to neutralization by aptamers or antibodies in the vitreous body[62].

An alternative option to modulate growth factor signaling is to inhibit the growth factor's receptor to prevent the intracellular downstream cascade, a principle which should not be affected by paracrine secretion or heparan sulfate proteoglycan binding[63-65]. Biochemically, VEGF and PDGF, as well as their receptors, share major ultrastructural similarities going so far that VEGF-A has been shown to be able to signal via PDGF receptors in mesenchymal stem cells[66]. Therefore, many tyrosine kinase inhibitors simultaneously modulating VEGF- and PDGF-receptor signaling are available[67]. For the experimental anti-VEGF/PDGF inhibition reported in Publication I and II herein, the tyrosine kinase inhibitor Axitinib (Inlyta; Pfizer, New York, USA) was chosen as it features the lowest half-maximal inhibitory concentration (IC_{50}) for VEGF receptors 1, 2 and 3 (0.1 – 0.3 nM) and PDGFR-ββ (1.6 nM) of all anti-VEGF/PDGF tyrosine kinase inhibitors available clinically; moreover, good biocompatibility with ocular tissue is suggesting it for intravitreal use[67]. While Axitinib also inhibits c-KIT, these effects on pericytes can be neglected as pericytes do not express c-KIT[68]. Axitinib has been approved by both the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) for the treatment of advanced renal cell carcinoma in 2012[69].

Publication I entitled "*Combined VEGF and PDGF inhibition for neovascular AMD: anti-angiogenic properties of axitinib on human endothelial cells and pericytes in vitro*" seeks to answer the question whether VEGF/PDGF-receptor inhibition using Axitinib poses a feasible alternative method of anti-angiogenesis against both effectors of CNV, namely endothelial cells and pericytes. Treating them with ascending doses of Axitinib, cellular angiogenic behaviour under VEGF- and PDGF-stimulation was evaluated in established models of angiogenesis.

Publication II entitled "*Combined VEGF/PDGF inhibition using axitinib induces alphaSMA expression and a pro-fibrotic phenotype in human pericytes"* is designed to evaluate the morphologic and structural changes in the target cell of anti-PDGF, the pericyte, in response to combined anti-VEGF/PDGF inhibition. Treating pericytes with Axitinib, its effects on pericyte cytoskeleton, mRNA and protein expression of pro-fibrotic α-smooth muscle actin (α-SMA) and pro-fibrotic behaviour were evaluated using established models of cellular wound healing.

4 Summary

In this *in vitro* model of nAMD, the influence of combined anti-VEGF/PDGF receptor inhibition on endothelial cells and pericytes was investigated. Both cell types are heavily involved in macular neovascularization in nAMD, which, left untreated or managed insufficiently, leads to atrophy and macular subretinal fibrosis, causing end stage subretinal fibrosis and severe vision loss[70,71]. Offering the lowest combined IC_{50} for VEGF receptors 1–3 and PDGF receptor ββ[72], providing good ocular biocompatiblity[72] and being approved by FDA and EMA for the use in humans[69], the tyrosine kinase inhibitor axitinib was used to assess the impact of anti-VEGF/PDGF on macular neovascularization and fibrotic damage resulting from angiogenesis.

In vitro, axitinib reduced VEGF-driven cellular mechanisms of angiogenesis, consisting of endothelial cell migration, ECM adhesion, subsequent proliferation and tube formation, resulting in a 112 % reduction of VEGF-induced tube length. While established anti-VEGF intravitreal therapy with Ranibizumab, Aflibercept or Bevacizumab is already offering these mechanisms of action, about 10-15 % of patients do not show an adequate response to current treatment[20,28,38]. For these patients, Axitinib shows a promising *in vitro* profile as a possible future alternative therapy modulating endothelial cell driven angiogenesis using growth factor receptor inhibition instead of growth factor elimination.

Moreover, axitinib allowed to expand the anti-angiogenetic spectrum beyond anti-VEGF towards PDGF and thus pericyte inhibition. PDGF driven pericyte proliferation and migration were significantly reduced, neutralizing the PDGF stimulated effect – causing a reversion of PDGF directed chemotaxis into a seemingly random chemokinesis, and inhibition of the PDGF driven proliferatory boost. A choroidal neovascularization (CNV) consisting of established endothelial tubes secretes PDGF to attract pericytes, which then in turn stabilize the tubes mechanically and via growth factor secretion[48]. By limiting pericyte proliferation and migration towards the CNV by blocking the PDGF pathway, axitinib might render endothelial cell-only CNV more vulnerable due to the lack of pericyte stabilization, which might prolong anti-VEGF action and force the endothelial tubes into regression. This way, clinical management of patients with nAMD would allow for a step towards CNV ablation instead of constant CNV persistence with constant regrowth after a drop of pharmacological compounds in the vitreous body, which at the moment requires continuous retreatment. Another additive effect of combined anti-VEGF/PDGF therapy observed was the reduction of

fibrovascular-like tissue surrounding endothelial tubes on basement membrane gels (up to -62 %). Scar material in macular fibrosis increases the more fibrovascular tissue a CNV generates; prevention of scarring thus requires a reduction of fibrovascular tissue[71]. As PDGF is a major player in fibrotic processes, from a future clinical perspective Axitinib treatment might reduce the fibrovascular burden accompanying CNV, clearing the subretinal space from debris causing photoreceptor disruption, atrophy, and visual loss[70,71].

On the contrary, from a clinical perspective the imagination of uncontrolled pericyte ablation might be a threat to the stability of healthy retinal vessels, as pericyte-drop out is a well-known first feature of diabetic microangiopathy[73]. In a second step, we therefore examined the influence of combined anti-VEGF/PDGF inhibition on pericyte morphology, dedifferentiation and cellular function – which is crucial, as steady endogenous PDGF levels are crucial to maintain endothelial-pericyte contact and crosstalk[44].

After 24 hours, pericytes treated with non-toxic concentrations of axitinib showed an increase in the actin cytoskeleton with focal densities already. As proven on messenger RNA and protein level, this change in phenotype is associated with a significant induction of α-smooth muscle actin. In other organs of the human body, mainly the lung and kidney, dedifferentiation of pericytes into a similar phenotype has been shown to be an important source of myofibroblasts, driving pathological fibrosis - a mechanism for which α-SMA upregulation, as in our case, is patho-gnomonic[51,68,74,75].

In our model, however, continued anti-VEGF/PDGF inhibition diminished the fibrotic behaviour of these transdifferentiated proto-myofibroblasts, namely scratch wound closure and collagen gel contraction. This might at first sound paradoxical, as migration and contraction are known to be promoted by an induction of contractile fibres of the cytoskeleton[76]. It has been shown, however, that both contraction[77,78] and migration[79,80] depend on signalling via phospholipase γ (PLC-γ), and contraction additionally on protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K)[77]. Therefore, continued upstream blockage of PDGF-BB receptors via axitinib seems to diminish the activation of the downstream effectors PLC-γ, PKC and PI3K, reducing the fibrotic processes mentioned above in spite of the phenotypic disposition of dedifferentiated pericytes to do so.

Criticism on our study might arise due to the fact that we used a VEGF/PDGF receptor tyrosine kinase inhibitor instead of an antibody or an aptamer, as used pre-clinically[41,54]. We however believe that this was the better choice as axitinib is approved clinically and has been proven effective in inhibiting VEGF and PDGF in humans[64,69]. In addition, it allowed for a more reproducible inhibition of PDGF signalling as PDGF mediating pericyte survival and attachment is secreted in a para/iuxtacrine fashion, and mostly bound to molecules around the pericyte itself, e.g. heparan sulphate proteoglycans; thus, vessel stabilizing, physiological PDGF is difficult to eliminate via antibodies or aptamers in cell culture medium[62]. The importance of this is also supported by the fact that exogenous PDGF *in vivo* does not support pericyte stability, but induces pericyte detachment from maturing vessels, as it is not delivered in the physiological para/iuxtacrine fashion[52].

In conclusion, this work strengthens the dichotomy of PDGF and PDGF inhibition: As PDGF is strongly involved in vascular formation and maintenance, PDGF inhibition represents a promising new strategy of retinal anti-angiogenesis by targeting pericyte-driven CNV stabilization. As, however, PDGF is pivotal for pericyte survival and differentiation, PDGF inhibition induces pericyte dedifferentiation towards a myofibroblastic phenotype[44,51]. Beyond its involvement in angiogenesis, in this case PDGF signalling takes on its pivotal role in fibrotic mesenchymal responses[62,68,81]. From a future clinical perspective of CNV management in nAMD, anti-PDGF tyrosine kinase activity via axitinib might be capable to reduce pericyte recruitment to growing CNV and strip pericytes off established CNV, rendering CNV more vulnerable to anti-VEGF. Additionally, axitinib might lead to a reduction of perivascular ECM formation around endothelial tubes, probably owing to the function of PDGF as modulator of the ECM[81]. On the other hand, PDGF deprived pericytes might undergo dedifferentiation into proto-myofibroblasts, resulting in an increase of pro-fibrotic cells in the macula[82]. These cells, however, seem to be less prone to fibrosis under continued anti-VEGF/PDGF combination treatment. Therefore, continued anti-VEGF/PDGF activity might be needed beyond its anti-neovascular properties to limit consecutive fibrotic responses. A similar switch between angiogenesis and fibrosis has been described for diabetic retinopathy, termed the ‘angiofibrotic switch’[83].

In summary, pericyte stabilization of CNV is hypothesized to be a major contributor to the high intensity and limited outcomes of state-of-the-art anti-VEGF therapy in nAMD[41,54]. In this context, our *in vitro* data advocate that a combined anti-VEGF/PDGF

intravitreal therapy might improve the management of pathologic neovascularization, prolonging treatment intervals and maybe also offering better long-term visual acuity. Further evidence, including *in vivo* animal studies are definitely needed. Figuratively speaking, however, combined VEGF/PDGF inhibition might offer the opportunity to tackle the CNV by the root, and not to just constantly prune it.

5 Zusammenfassung (deutsche Version)

Anhand eines *in vitro* Zellkulturmodells der neovaskulären AMD (nAMD) wurde in dieser Arbeit untersucht, welchen Einfluss eine kombinierte Inhibition der beiden Wachstumsfaktoren *vascular endothelial growth factor* (VEGF) und *platelet derived growth factor* (PDGF) auf Endothelzellen und Perizyten ausübt. Beide Zelltypen sind verantwortlich für die Bildung makulärer choroidaler Neovaskularisationen (CNV), die unbehandelt zum Endstadium der nAMD, der submakulären Fibrose, und einer daraus resultierenden hochgradigen Sehminderung führen[70,71]. Um den Effekt einer kombinierten VEGF/PDGF-Inhibition auf makuläre Neovaskularisationen und die aus der Angiogenese resultierende Fibrose zu untersuchen, wurde der Tyrosinkinase-Inhibitor Axitinib ausgesucht. Axitinib eignet sich für diese Fragestellung besonders, da diese Substanz die niedrigste klinisch verfügbare mittlere inhibitorische Konzentration (IC_{50}) für die VEGF-Rezeptoren 1–3 und den PDGF ββ-Rezeptor[72] bietet, eine gute okuläre Biokompatibilität aufweist[72], und nicht zuletzt durch die FDA und EMA für den klinischen Einsatz am Menschen zugelassen ist[69].

In vitro führte Axitinib zu einer signifikanten Reduktion maßgeblicher endothelialer Angiogeneseprozesse, nämlich der Endothelzellmigration, der Adhäsion, Proliferation und Bildung kapillarähnlicher Fortsätze (*tube formation).* Die Länge dieser kapillarähnlichen Fortsätze (*„tubes“)*, die unter VEGF-Stimulation auf Basalmembran-Gelen untersucht wurde, zeigte sich nach Behandlung mit Axitinib um 112 % reduziert. Obwohl eine ähnliche Wirksamkeit auch von den im klinischen Einsatz befindlichen Substanzen Ranibizumab, Aflibercept und Bevacizumab in solch einem experimentellen Aufbau zu erwarten ist, bieten unsere *in vitro*-Daten Hinweise, dass Axitinib eventuell als Therapiealternative für die 10-15 % der Patienten in Frage käme, die in der Literatur als Non-Responder auf den heutigen Therapiestandard – nämlich reine Wachstumsfaktorinhibition – gelten[20,28,38]. Für diese Patienten zeigt Axitinib ein vielversprechendes *in vitro*-Profil, um die durch Endothelzellen vermittelte Angiogenese nicht nur mittels Wachstumsfaktor-Blockade, sondern mittels entsprechender Rezeptormodulation einzuschränken.

Weiterhin erlaubte es Axitinib als breit wirksamer Tyrosinkinaseinhibitor, das pharmakologische Spektrum der Behandlung für die nAMD über anti-VEGF hinaus zu erweitern. Anders als anti-VEGF-Hemmer im klinischen Einsatz ist Axitinib auch gegen den Wachstumsfaktor PDGF wirksam, der in der Angiogenese ein wichtiges Signalmolekül für Zellen der Gefäßwand, hauptsächlich Perizyten darstellt. Während

der Entstehung einer CNV bilden Endothelzellen zunächst Kapillaren, die in der Folge PDGF sezernieren, um Perizyten zu rekrutieren; diese stabilisieren die neuen Kapillaren mechanisch und mittels Wachstumsfaktorsekretion, um sie damit vor einer Rückbildung durch Entzug von VEGF zu schützen[48]. *In vitro* konnte Axitinib sowohl die PDGF-abhängige Proliferation, als auch Migration der Perizyten erfolgreich inhibieren. Da PDGF den wichtigsten Proliferationsimpuls und das wichtigste Chemokin im Rahmen dieser Rekrutierung von Perizyten Richtung CNV darstellt, könnte Axitinib eventuell auch in Zukunft *in vivo* die gerichtete Chemotaxis zur CNV in eine ungerichtete Chemokinesis umwandeln, und die Zahl der Perizyten auf der sich bildenden CNV deutlich verringern. Somit könnte Axitinib die nur aus Endothelzellen bestehende CNV deutlich anfälliger für anti-VEGF machen, da keine Perizyten-abhängige mechanische und über Wachstumsfaktoren vermittelte Stabilisierung des entstehenden Gefäßes bestünde. Auf diese Weise könnte eine kombinierte anti-VEGF/PDGF-Inhibition in Zukunft eine echte Rückbildung der CNV ermöglichen, während unter Therapie mit den bisherigen anti-VEGF-Substanzen von Perizyten besetzte CNV-Gefäßstümpfe persistieren, aus denen neue Kapillaren sprossen, sobald die anti-VEGF-Wirkspiegel im Glaskörper sinken. Die Notwendigkeit der stetigen Wiederbehandlung könnte somit in Zukunft potentiell vermieden werden.

Als weiterer Zusatznutzen gegenüber anti-VEGF als Monotherapie zeigte sich, dass Axitinib die Menge fibrovaskulären Gewebes um kapilläre endotheliale Strukturen auf Basalmembran-Gelen um bis zu 62 % reduzieren konnte. Um eine makuläre Fibrose zu verhindern, muss die Bildung fibrovaskulären Materials reduziert werden[71]. Da PDGF stark in Signalwege involviert ist, die fibrotisches Material produzieren, könnte Axitinib in Zukunft die Fibrosierung einer CNV reduzieren. Dadurch könnten Photorezeptoren und damit Sehvermögen besser besser erhalten bleiben[70,71].

Perizyten sind für eine gesunde Netzhautvaskulatur unerlässlich; deshalb ist auf der anderen Seite eine unkontrollierte PDGF-Deprivation retinaler Perizyten an gesunden Gefäßen streng zu vermeiden, da ein Perizytenverlust eines der ersten Zeichen der diabetischen Mikroangiopathie darstellt[73]. Aus diesem Grund untersuchten wir in einem zweiten Schritt, welche Auswirkung eine kombinierte anti-VEGF/PDGF-Inhibition auf die perizytäre Morphologie, Dedifferenzierung und Zellfunktion hatte. Dies ist klinisch hochrelevant, da dauerhafte endogene PDGF-Level vonnöten sind, um die physiologische Zusammenarbeit von Endothelzelle und Perizyt sicherzustellen[44].

Weniger als 24 Stunden nach einer Behandlung mit nicht-toxischen Dosen von Axitinib zeigte sich in den Perizyten schon eine Induktion des Aktin-Zytoskeletts mit fokalen Zytoskelett-Verdichtungen. Wir konnten auf mRNA- und Proteinebene bestätigen, dass diese Umbauvorgänge mit einer signifikanten Induktion von α-smooth muscle Aktin (αSMA) assoziiert sind. In anderen Organen des menschlichen Körpers, hauptsächlich der Lunge und Niere, wurde bereits nachgewiesen, dass solch dedifferenzierte Perizyten eine Hauptquelle von Myofibroblasten darstellen, die zu einer pathologischen, überschüssigen Fibrose führen; für diesen Mechanismus ist eine Hochregulation von αSMA pathognomonisch[51,68,74,75].

Trotz dieser Änderung des Phänotyps kam es in den nachfolgenden Zellversuchen zu einer Reduktion pro-fibrotischer Zellmechanismen im *scratch wound*- und Kontraktionsassay. Dies mag zunächst paradox klingen, da beide Mechanismen von einer Hochregulierung kontraktiler Fasern begünstigt werden[76]. Es wurde allerdings gezeigt, dass zelluläre Kontraktion[77,78] und Migration[79,80] von der Signalweiterleitung mittels Phospholipase γ (PLC-γ), und Kontraktion alleine zusätzlich von einer Signalweiterleitung mittels Proteinkinase C (PKC) und Phosphoinositide 3-kinase (PI3K) abhängen[77]. Daher ist anzunehmen, dass eine Blockade des PDGF-Signalweges die Aktivierung von PLC-γ, PKC und PI3K verringert, was zu einer Reduktion oben genannter fibrotischer Prozesse führt, obwohl die Zellmorphologie sich pro-fibrotisch präsentierte.

An unserer Studie mag zu kritisieren sein, dass wir statt eines präklinischen anti-PDGF-Antikörpers oder Aptamers einen Tyrosinkinase-Inhibitor nutzten[41,55]. Dies ist allerdings dadurch zu rechtfertigen, dass Axitinib bereits zugelassen ist und in anderen *in vitro* Modellen der okulären Angiogenese gut funktioniert[64,69]. Zusätzlich ist zu bedenken, dass das PDGF, das zum Überleben und zur Kommunikation zwischen Endothelzelle und Perizyt beiträgt, para/iuxtakrin sezerniert wird und zu Großteilen an Heparansulfate um den Perizyten gebunden ist; dadurch ist es schwer durch Antikörper oder Aptamere im Zellkulturmedium zu eliminieren[62]. Dies wird auch dadurch bekräftigt, dass *in vivo* exogenes PDGF nicht zur Stabilisierung des Perizyten beiträgt, sondern Perizyten von Gefäßwänden ablöst, da es nicht in der physiologischen para/iuxtakrinen Art sezerniert wird[52].

Zusammenfassend stärkt unsere Arbeit die Dichotomie von PDGF und PDGF-Inhibition. Da PDGF essentiell für Angiogenese und vaskuläre Homöostase ist,

repräsentiert die PDGF-Inhibition eine vielversprechende Strategie gegen CNV bei nAMD. Da PDGF aber den wichtigsten Wachstumsfaktor für das Überleben und die Differenzierung von Perizyten darstellt, führt eine PDGF-Inhibition zur perizytären Dedifferenzierung in Richtung Myofibroblast[44,51]. In diesem Fall rückt die Rolle von PDGF als Modulator fibrotischer mesenchymaler Gewebereaktionen in den Vordergrund[62,68,81]. In einem möglichen zukünftigen klinischen Einsatz könnte Axitinib über eine zusätzliche PDGF-Inhibition die Rekrutierung von Perizyten zur CNV stoppen, CNV-assoziierte Perizyten von der CNV lösen, und dadurch die CNV intensiver gegenüber anti-VEGF exponieren. Zusätzlich könnte Axitinib die Menge des perivaskulären Gewebes reduzieren, das endotheliale Gewebestrukturen umgibt, da PDGF in seiner Funktion als Modulator der extrazellulären Matrix (ECM) gehemmt wird[81]. Auf der anderen Seite erfolgt als Reaktion auf den pharmakologischen PDGF-Entzug eine Dedifferenzierung der Perizyten in Richtung proto-Myofibroblast, was die pro-fibrotische Zelllast in der Makula erhöht[82]. Wird die anti-VEGF/PDGF-Therapie jedoch fortgeführt, zeigt dieser Zelltyp eine untypischer Weise geringe Aktivierung pro-fibrotischer Zellprozesse. Deshalb könnte die kombinierte anti-VEGF/PDGF-Therapie im klinischen Einsatz über ihre anti-angiogene Funktion hinaus eine große Rolle spielen, um aus Angiogenese resultierende fibrotische Prozesse zu modulieren, wie man es vom ‘angiofibrotischen switch’ in der diabetischen Retinopathie her kennt[83].

Es ist davon auszugehen, dass die perizytäre Stabilisierung der CNV zur hohen Therapieintensität der derzeitigen anti-VEGF-Therapie und bisher limitierten Langzeit-Visusprognose beiträgt[41,54]. In diesem Zusammenhang machen unsere *in vitro*-Daten Hoffnung, dass Axitinib möglicherweise auch klinisch in Zukunft längere Therapieintervalle und einen besseren Erhalt der Sehfunktion zulassen könnte. Weitere Studien, insbesondere im Tiermodell sind nötig, um die breite Anwendbarkeit weiter zu prüfen. Eine kombinierte VEGF/PDGF-Inhibition könnte jedoch die Möglichkeit bieten, über die Induktion einer vaskulären Regression die CNV kausal zu therapieren, statt nur mit wiederholten Re-Injektionen einen kurzfristigen Effekt auf das erneute Gefäßwachstum auszuüben.

6 Publications resulting from this thesis

I.

Siedlecki J, Wertheimer C, Wolf A, Liegl R, Priglinger C, Priglinger S, Eibl-Lindner, K. Combined VEGF and PDGF inhibition for neovascular AMD: anti-angiogenic properties of axitinib on human endothelial cells and pericytes in vitro.
Graefe's Archive for Clinical and Experimental Ophthalmology. 2017;255(5):963-972
doi: 10.1007/s00417-017-3595-z

II.

Siedlecki J, Asani B, Wertheimer C, Hillenmayer A, Ohlmann A, Priglinger C, Priglinger S, Wolf A, Eibl-Lindner K. Combined VEGF/PDGF inhibition using axitinib induces alphaSMA expression and a pro-fibrotic phenotype in human pericytes.
Graefe's Archive for Clinical and Experimental Ophthalmology. 2018;256(6):1141-1149
doi: 10.1007/s00417-018-3987-8

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9 Curriculum vitae

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CURRICULUM VITAE

Person

Nationality:	German

School Education

2008	High School graduation from Gymnasium bei St. Stephan, Augsburg (final grade: „very good: 1.0“)

Studies and professional training

2008 – 2010	Medical Studies at Ludwig Maximilians-University, Munich
2010 – 2015	Medical Studies at Technical University, Munich
07/2015	Medical license, graduating from Technical University Munich (final grade: „very good: 1.16“)
since 09/2015	Resident in Ophthalmology at the University Eye Hospital Munich

Professional societies

2014	Association for Research in Vision and Ophthalmology (ARVO)
since 2016	German Ophthalmological Society (DOG)
since 2017	European Society of Cataract and Refractive Surgeons (ESCRS)

Training abroad

08/2011	Landspítali University Hospital, Reykjavík, Island
09 – 12/2014	Inselspital University Hospital, Bern, Switzerland

Research (please also see the publication list attached)

since 04/2013	Experimental research at the Laboratory of cellular biology, University Eye Clinic Munich
since 09/2015	Clinical research (anterior segment and retina) at the University Eye Clinic Munich and the SMILE Eyes Clinic Linz, Austria
since 2017	Reviewer for *Journal of Ophthalmology*, *Microcirculation*, *European Journal of Ophthalmology*

Awards and Scholarships

02/2017	Best Presentation Award of the *German Society for Intraocular Lens Implantology (DGII)*
2016 – 2017	*Friedrich Baur Foundation* Research Grant for the in-vitro evaluation of combined endothelial cell/pericyte inhibition for neovascular AMD
06/2016	Travel Grant of the *German Retina Society*
2008 – 2015	Full study scholarship of the *German National Merit Foundation* („Studienstiftung des deutschen Volkes“)
2008 – 2015	Full study scholarship of the *Bavarian Merit Foundation* („Max Weber-Programm“)

10 Publication list

A) Publications with peer review

1. **Siedlecki J**, Luft N, Keidel L, Mayer WJ, Kreutzer TC, Priglinger SG, Archer TJ, Reinstein DZ, Dirisamer M. Variation of lenticule thickness for SMILE in Low Myopia. *Journal of refractive surgery.* 2018;34(7):453-459

2. Luft N, Schumann RG, Dirisamer M, Kook D, **Siedlecki J**, Wertheimer C, Priglinger SG, Mayer WJ. Wound Healing, Inflammation, and Corneal Ultrastructure After SMILE and Femtosecond Laser-Assisted LASIK: A Human Ex Vivo Study. *Journal of refractive surgery.* 2018;34(6):393-399.

3. Wertheimer C, Kueres A, **Siedlecki J**, Braun C, Kassumeh S, Wolf A, Mayer W, Priglinger C, Priglinger S, Eibl-Lindner K. The intraocular lens as a drug delivery device for an epidermal growth factor-receptor inhibitor for prophylaxis of posterior capsule opacification. *Acta Ophthalmologica*. 2018. doi: 10.1111/aos.13759. [Epub ahead of print]

4. **Siedlecki J**, Asani B, Wertheimer C, Hillenmayer A, Ohlmann A, Priglinger C, Priglinger S, Wolf A, Eibl-Lindner K. Combined VEGF/PDGF inhibition using axitinib induces alphaSMA expression and a pro-fibrotic phenotype in human pericytes. *Graefe's Archive for Clinical and Experimental Ophthalmology.* 2018;256(6):1141-1149

5. **Siedlecki J**, Luft N, Mayer WJ, Siedlecki M, Kook D, Meyer B, Bechmann M, Wiltfang R, Priglinger SG, Dirisamer M. CIRCLE Enhancement After Myopic SMILE. *Journal of refractive surgery.* 2018;34(5):304-309.

6. Luft N, **Siedlecki J**, Sekundo W, Wertheimer C, Kreutzer TC, Mayer WJ, Priglinger SG, Dirisamer M. Small incision lenticule extraction (SMILE) monovision for presbyopia correction. *European journal of ophthalmology.* 2018;28(3):287-293.

7. Schumann RG, Vogt D, Haritoglou C, Hagenau F, **Siedlecki J**, Wolf A, Priglinger SG. [Histopathological correlation of epiretinal tissue in lamellar macular holes and macular pseudoholes]. *Der Ophthalmologe.* 2017;114(12):1110-1116.

8. **Siedlecki J**, Luft N, Kook D, Wertheimer C, Mayer WJ, Bechmann M, Wiltfang R, Priglinger SG, Sekundo W, Dirisamer M. Enhancement after myopic small incision lenticule extraction (SMILE) using surface ablation. *Journal of refractive surgery.* 2018;33(8):513-518

9. **Siedlecki J**, Mackert MM, Wolf A, Berking C, Priglinger SG, Eibl-Lindner K. Bilateral visual field defects in a patient treated with Trametinib and Dabrafenib for melanoma of unknown origin. *Retinal Cases and Brief Reports. 2017 Apr [Epub ahead of print]*

10. **Siedlecki J,** Reiterer V, Leicht S, Foerster S, Kortüm K, Schaller U, Priglinger SG, Fuerweger C, Muacevic A, Eibl-Lindner K**.** Incidence of secondary

glaucoma after treatment of uveal melanoma with robotic radiosurgery versus brachytherapy. *Acta Ophthalmologica. 2017;95(8):e734-e739.*

11. **Siedlecki J,** Wertheimer C, Wolf A, Liegl R, Priglinger C, Priglinger S, Eibl-Lindner, K. Combined VEGF and PDGF inhibition for neovascular AMD: anti-angiogenic properties of axitinib on human endothelial cells and pericytes in vitro. *Graefe's Archive for Clinical and Experimental Ophthalmology.* 2017;255(5):963-972

12. Wertheimer C, **Siedlecki J**, Kook D, Mayer W, Wolf A, Klingenstein A, Kampik A, Eibl-Lindner K: EGF-Receptor Inhibitor Gefitinib attenuates posterior capsule opacification in vitro and in the ex vivo human capsular bag model. *Graefe's Archive for Clinical and Experimental Ophthalmology. 2015;253(3): 409-17*

13. Liegl R, Koenig S, **Siedlecki J**, Haritoglou C, Kampik A, Kernt M: Temsirolimus inhibits proliferation and migration in retinal pigment epithelial and endothelial cells via mTOR inhibition and decreases VEGF and PDGF expression. *PLoS One. 2014 Feb 26;9(2): e88203.*

14. Thiele S, Liegl RG, König S, **Siedlecki J**, Langer J, Eibl K, Haritoglou C, Kampik A, Kernt M: [Multikinase-Inhibitors as new therapy option in neovascular AMD: In-vitro evaluation of the intravitreal safety-profile of Axitinib, Pazopanib and Sorafenib]. *Klinische Monatsblätter für Augenheilkunde. 2013; 230(3): 247-254*

B) Citeable conference abstracts

1. EURETINA 2018, Vienna
   Siedlecki J, Vounotrypidis E, Vogt D, F. Hagenau F, Wolf A, Priglinger S, Schumann R: Simultaneous MacTel type 1 and lamellar hole associated epiretinal proliferation detected by multimodal imaging

2. WORLD OPHTHAMOLOGY CONFERENCE 2018, Barcelona
   Siedlecki J, Luft N, Priglinger S, Dirisamer M: CIRCLE enhancement after myopic small incision lenticule extraction (SMILE)

3. ESCRS 2017, Lisbon
   Siedlecki J, Luft N, Kook D, Luft N, Wiltfang R, Dirisamer M: Enhancement after myopic small incision lenticule extraction (SMILE) using surface ablation.

4. EURETINA 2016, Copenhagen
   Siedlecki J, Wertheimer C, Wolf A, Alge-Priglinger C, Priglinger SG, Eibl-Lindner EK: Axitinib exerts anti-angiogenic properties on human endothelial cells and pericytes in an in vitro-model of nAMD via combined VEGF and PDGF tyrosine kinase inhibition.

5. GERMAN OPHTHALMOLOGICAL SOCIETY 2015, BERLIN
   Siedlecki J, Wertheimer C, Liegl R, Kernt M, Kampik A, Eibl-Lindner EK:
   [The pericyte as new target in anti-neovascular therapy: Inhibition of proliferation and migration of human pericytes after treatment with the tyrosine kinase inhibitor Axitinib]

6. WORLD OPHTHAMOLOGY CONFERENCE 2014, TOKYO
   Siedlecki J, Liegl R, Kernt M, Arend N, Kampik A: Axitinib Inhibits Vascular Endothelial Growth Factor (VEGF) Expression and Secretion and Modulates Anti-VEGF Induced Dysregulation of VEGF/Connective Tissue Growth Factor (CTGF) Ratio in Human Retinal Pigment-Epithelium and Vascular Endothelial Cells

C) Publications for magazines without peer review

1. **Siedlecki J.** Evaluating Corneal Refractive Options for Presbyopic Patients: Efficacy and Patient Satisfaction. *EuroTimes Supplement, September 2018*

2. **Siedlecki J.** SMILE monovision – an effective and safe treatment for presbyopic ametropic patients. *Ophthalmology Times, August 2018*

3. **Siedlecki J.** [Nachkorrektur nach myoper SMILE mittels PRK]. *DGII Aktuell, October 2017*

4. **Siedlecki J,** Luft N, Dirisamer M. [Advanced Triple Procedure: DMEK bei komplexen Vorderabschnittspathologien]. *Der Augenspiegel, October 2017*

5. **Siedlecki J**. [Fibrosis and atrophy: still unsolved long-term complications of neovascular AMD]. *Ophthalmologische Nachrichten, April 2017*

6. **Siedlecki J**, Schumann RG. [Paradigm shift in neovascular AMD]. *Ophthalmologische Nachrichten, April 2017*