INHIBITORS OF CORNEAL INFLAMMATION AND ANGIOGENESIS: PROSPECTIVES AND CHALLENGES

Pierfrancesco Mirabelli

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Linköping 2019

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Linköping University Medical Dissertations, No. 1685

Published by Linköping University

Printed by LiU-Tryck, Linköping, Sweden, 2019

ISBN: 978-91-7685-064-0

ISSN: 0345-0082

Inhibitors of Corneal Inflammation and Angiogenesis:

Prospectives and Challenges

ACADEMIC THESIS By Pierfrancesco Mirabelli

FOR THE AWARD OF A DOCTORATE DEGREE (Ph.D.)

By

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Examination held at

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Friday 17th of May 2019, 13:00 hrs

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...Instruction, and not silver; and Knowledge rather than choice gold.

For Wisdom is better than rubies...

(Proverbs 8: 10-11)

ABSTRACT

Pathologic angiogenesis is involved in cancer and several blinding conditions such as wet age-related macular degeneration, proliferative retinopathies and corneal neovascularization.

In these diseases, the angiogenic triggers are hypoxia and inflammation, and both involve the main angiogenic mediator, which is Vascular Endothelial Growth Factor (VEGF). Among available treatments, anti-VEGF often shows limited or temporary efficacy, while steroids are potentially responsible for many side-effects. This thesis presents a series of linked studies aimed at elucidating the early pathologic changes leading to inflammation and corneal neovascularization, and how various treatments affect this process. In this thesis, anti-inflammatory and anti-angiogenic treatments are applied in corneal neovascularization models, to identify VEGF-independent pathways and other novel factors as future therapy targets, as well as to investigate the endogenous modulation of angiogenesis.

A model of experimental neovascularization in the rat cornea was used as main model, where the neovascular response is triggered by a surgical suture placed into the cornea. Investigational treatments (anti-Vegf, dexamethasone, IMD0354, Gap27, or control substances) were then given topically, with the exception of IMD0354, which was given systemically. The effects in the cornea were studied *in vivo* with slit lamp photography to assess and quantify macroscopic vessel growth and using *in vivo* confocal microscopy (IVCM) to study cell infiltration and limbal vessel dilation and detect microscopic vessel sprouts; these examinations were performed longitudinally. Genomic analysis with RNA microarray, selected gene expression with q-RT-PCR, and selected protein expression in tissue (immunohistochemistry, immunofluorescence, Western blot) were performed at different time-points. Moreover, other experiments on cell cultures (HUVEC and HCEC), organ cultures (human

corneas), *ex vivo* models (aortic rings) and *in vivo* studies (zebrafish vasculogenesis) were performed.

Dexamethasone suppressed limbal vasodilation and corneal neovascularization more than anti-Vegf, despite no difference in inflammatory cell infiltration into the cornea. Five-hundred eleven fewer genes were differentially expressed in dexamethasone-treated corneas relative to naïve corneas, compared to anti-Vegf. Among them, several major pro-angiogenic and pro-inflammatory factors and chemokines were suppressed only by dexamethasone and represent novel candidate factors to target in order to improve anti-VEGF treatment. On the other hand, selective inhibition of a single inflammatory pathway (NF- κ B), despite showing similar early effects as dexamethasone in suppressing tissue inflammation, was not effective enough to suppress new vessel growth. The same factors suppressed by dexamethasone are also inhibited in endogenous modulation of angiogenesis. Surprisingly, dexamethasone activated several complement factors, which could possibly be beneficial in the anti-angiogenic response.

In a different therapeutic approach, promoting cell migration to accelerate epithelial wound closure similarly was not sufficient to avoid inflammation and angiogenesis in the cornea.

In conclusion, new and more effective treatments are needed for corneal inflammation and neovascularization with fewer side-effects. In this thesis, several novel factors and mechanisms related to inflammation are identified, factors that are not addressed by anti-Vegf therapy, and therefore represent interesting objects for further study, as they have the potential to be targets for adjuvant therapy. Specific anti-inflammatory treatment as well as therapeutic activation of endogenous regulatory pathways, and potentially complement modulation, might represent new strategies to improve anti-angiogenic therapy, but when used alone they do not seem to avoid corneal neovascularization.

SAMMANFATTNING

Angiogenes betyder kärltillväxt från befintliga kärl, och är en viktig fysiologisk process i fosterstadiet, muskeltillväxt, sårhäkning och menstruation. En okontrollerad tillväxt, en sjuklig kärlnybildning är dock involverad i många sjukdomar, som cancer, hjärtsjukdomar och ögonsjukdomar. I ögat kan en okontrollerad kärltillväxt skada synen: 1) våta åldersförändringar i gula fläcken är den främsta orsaken till allvarlig synnedsättning i västvärlden; 2) näthinnan och dess synceller kan skadas även av kärl som växer på grund av diabetes eller blodproppar; 3) hornhinnan är ögats genomskinliga fönster, och kärl som växer här till följd av infektion, sår, frätskada kan orsaka ärrbildning och blindhet. Alla dessa sjukdomar har som gemensam nämnare syrebrist och inflammation, som båda stimulerar produktion av den viktigaste faktorn som driver kärltillväxt, Vascular Endothelial Growth Factor (VEGF). Kärlnybildning i hornhinnan behandlas ofta med kortison, som kan orsaka biverkningar såsom grönstarr, gråstarr, och hornhinnesår. På senare år har man börjat använda anti-VEGF behandling med läkemedel som specifikt hämmar VEGF och som har blivit standardbehandlingen för våta åldersförändringar i gula fläcken eller diabetesförändringar; dock har denna behandling ofta kortvarig, begränsad, eller utebliven effekt. Troligen förklaras detta av VEGF-oberoende kaskader som inte hämmas av denna behandling, molekylära kaskader som ännu inte är väl kända. Likaså okänt är hur anti-VEGF och steroider (kortison) påverkar angiogenesens tidiga skeden.

Denna avhandling presenterar en serie studier som är syftade till att klargöra i detalj tidiga skeenden i kärlnybildningsprocessen; vidare, hur processen påverkas av kortisonbehandling (dexametason), anti-VEGF behandling, och av två andra experimentella behandlingar: en specifik antinflammatorisk behandling (IMD) samt en specifik behandling för snabbare sårhäkning (Gap27). Alla dessa behandlingar har givits som ögondroppar förutom den tredje (IMD).

Vi använder oss av en djurmodell av inflammatorisk angiogenes i hornhinna i råtta där kärlnybildningen stimuleras genom placering av stygn; man följer sen processen utan eller med behandlingarna. Studien baseras på undersökningar *in vivo* som mikroskop och spaltlampefotografering; bland dessa undersökningar, IVCN (in vivo confocal microscopy), som tillåter visualiseringen av levande vävnad på cellnivå. Vidare har vi studerat förändringar i vävnaden på molekylnivå och gennivå genom genuttrycksanalys med microarray, som möjliggör analysen av tusentals gener för att hitta vilka av dessa påverkas av processen och av behandlingen; utvalda gener har analyserats även med andra laborietekniker som qRT-PCR (Real Time Polymerase Chain Reaction), Western blot (proteinanalys) och vävnadsinfärgningar.

I studie I har vi observerat att kortison droppar blockerar kärlnybildning i hornhinnan mycket bättre än anti-VEGF. Vid mikroskopundersökning, innan kärlen blir synliga, ser man att kortison också blockerar vidgningen av de redan befintliga kärlen i området runt hornhinnan (limbus), vidgning som annars observeras i processen i alla andra grupper. Ingen skillnad mellan behandlingar har setts i tillkomst av inflammatoriska celler (vita blodkroppsceller) som infiltrerar vävnaden, förutom viss försening av en specifik sort celler (makrofager) som syns i kortisonbehandlingen. Frågan som drivit studierna vidare har varit: ”vad är det som orsakar den stora skillnaden i observerad effekt, alltså i hämning av kärltillväxt, om ingen signifikant skillnad har kunnat observeras i antal inflammatoriska celler som strömmar in?”. Den breda analysen av genuttrycket som gjorts i studie II har då visat att kortisonbehandling påverkar många fler gener än anti-VEGF behandlingen. Kortison hämmar många gener som är inblandade i inflammationen, och som skulle kunna vara mål för att utveckla nya behandlingar. Kortison har också visat sig stimulera en grupp faktorer som förknippas med inflammationen, som hör till den så kallade ”komplementkaskaden”, och som förmodligen kan hjälpa

till att bekämpa kärlnybildningen; dessutom, kan komplementkaskaden troligtvis förklara att så många celler strömmar in trots att många faktorer som har som funktion att kalla in celler är nertryckta av behandlingen.

Kortison har en mycket bred effekt, vilket också kan förklara de många biverkningarna. En mer specifik behandling som hämmar den inflammatoriska kaskaden, huvudorsaken till kärlnybildning i hornhinnan, har testats i studie III, för att se om man kan ha minska biverkningarna och ändå ha bra effekt. Trots en effekt som mikroskopiskt (IVCM visade hämning av kärlvidgning i limbus men ingen hämning av vita blodkropparna som kommer in i vävnaden) och i genuttrycket (samma inflammationsfaktorer hämmas som vid kortison) liknar kortison, så har kärlen ändå vuxit in i hornhinnan. Det betyder att hämning av bara en specifik inflammationskaskad inte är tillräcklig som behandling, utan att detta bör förmodligen associeras med till exempel anti-VEGF behandlingen. Studie IV undersöker om en behandling som snabbar på sårläkningsprocessen kan aktivera kroppens egna processer som motverkar kärlnybildningen: såret läkte fortare men det gick inte att undvika kärltillväxt.

Sammanfattningsvis, nya mer effektiva behandlingar med färre biverkningar behövs för kärlnybildning i hornhinnan. I denna avhandling presenteras flera nya molekylära faktorer kopplade med inflammationen, som inte påverkas av anti-VEGF behandlingen, och som skulle kunna användas för att utveckla nya behandlingar. En framgångsrik behandlingsstrategi skall baseras på hämningen av flera olika faktorer samtidigt, och eventuellt även på stimuleringen av kroppens egna kontrollmekanismer. Ny forskning behövs för att studera i detalj effekten av hämningen av dessa faktorer som här föreslås; vidare bör man testa om samma koncept kan appliceras till andra vävnader där effektivare behandlingar behövs för många synhotande sjukdomar och potentiellt även livshotande tillstånd

LIST OF PUBLICATIONS INCLUDED IN THE THESIS

PAPER I

Early effects of dexamethasone and anti-VEGF therapy in an inflammatory corneal neovascularization model. Mirabelli P, Peebo BB, Xeroudaki M, Koulikovska M, Lagali N. Exp Eye Res. 2014 Aug;125:118-27. doi:10.1016/j.exer.2014.06.006.

PAPER II

Genome-wide expression differences in anti-Vegf and dexamethasone treatment of inflammatory angiogenesis in the rat cornea. Mirabelli P, Mukwaya A, Lennikov A, Xeroudaki M, Peebo B, Schaupper M, Lagali N. Sci Rep. 2017 Aug 15;7(1):7616. doi:10.1038/s41598-017-07129-4.

PAPER III

Selective IKK2 inhibitor IMD0354 disrupts NF- κ B signaling to suppress corneal inflammation and angiogenesis. Lennikov A, Mirabelli P, Mukwaya A, Schaupper M, Thangavelu M, Lachota M, Ali Z, Jensen L, Lagali N. Angiogenesis. 2018 May;21(2):267-285. doi:10.1007/s10456-018-9594-9.

PAPER IV

Effect of connexin 43 inhibition by the mimetic peptide Gap27 on corneal wound healing, inflammation and neovascularization. Elbadawy HM, Mirabelli P, Xeroudaki M, Parekh M, Bertolin M, Breda C, Cagini C, Ponzin D, Lagali N, Ferrari S. Br J Pharmacol. 2016 Oct;173(19):2880-93. doi:10.1111/bph.13568.

Publication related to Paper II (not included in the thesis)

Genome-wide expression datasets of anti-VEGF and dexamethasone treatment of angiogenesis in the rat cornea. Mukwaya A, Mirabelli P, Lennikov A,

Xeroudaki M, Schaupper M, Peebo B, Lagali N. Sci Data. 2017 Aug 15; 4:170111. doi:10.1038/sdata.2017.111.

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ABBREVIATIONS

ACAID	Anterior Chamber Immuno Deviation
AMD	Age-related Macular Degeneration
ARVO	The Association for Research in Vision and Ophthalmology
CCL	Chemokine C-C motif Ligand
CD	Cluster of Differentiation
cDNA	Complementary DNA
CNV	Choroidal neovascularization
CX43	Connexin 43
cRNA	Complementary RNA
CXCL	Chemokine C-X-C motif Ligand
DAMP	Damage Associated Molecular Pattern
DAPI	4', 6-diamidino-2-phenylindole
DEG	Differentially Expressed Gene
DME	Diabetic Macular Edema
DR	Diabetic Retinopathy
EC	Endothelial Cell
ECM	Extracellular Matrix
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HE	Hematoxilin Eosin

HCECs	Human Corneal Epithelial Cells
HIF	Hypoxia Inducible Factor
HUVECs	Human Umbilical Vein Endothelial Cells
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IVCM	In vivo confocal microscopy
MCP	Monocyte Chemotactic Protein
MMP	Matrix MetalloProteinase
NF	Nuclear Factor
NO	Nitric Oxyde
OCT	Optical Coherence Tomography
OIR	Oxygen Induced Retinopathy
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PEDF	Pigment Epithelium Derived Growth Factor
PIGF	Placental Growth Factor
qRTPCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic Acid
ROP	Retinopathy of Prematurity
RVO	Retinal Vein Occlusion

TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF Receptor

NOMENCLATURE FOR GENES AND PROTEINS

Genes are reported with italics (for example *Vegfa*).

Proteins are reported with normal characters (for example VEGF or Vegf, see down).

Human genes and proteins are reported with bold characters (for example **VEGF**).

Animal genes and proteins are reported with small characters (for example **Vegf**).

1 INTRODUCTION AND BACKGROUND

Many blinding eye diseases are still a challenge for therapy today: age-related macular degeneration (AMD) and diabetic retinopathy (DR) are the main causes of visual impairment in the Western world across all ages ¹, and in the population under 50 years of age ², respectively. Corneal scarring is the fourth largest cause of blindness globally after cataract, glaucoma and AMD ³. AMD, DR and corneal scarring share a common denominator, which is represented by inflammation leading to a pathologic growth of new vessels, a process termed “angiogenesis” (from the greek *angios* and *gigno*). Pathologic angiogenesis is also involved in other ophthalmic diseases (uveitis, that is ocular inflammatory diseases, and retinopathy of prematurity, ROP), as well as in cardiovascular diseases, systemic inflammatory pathologies, and cancer ⁴. Corneal blindness can be caused by trauma and ulceration, or more often by corneal inflammation due to infections, such as trachoma, frequent in developing countries, or herpes virus ^{3,5,6}.

This thesis examines the processes of inflammation and angiogenesis in disease and how various treatments affect them, with special focus on corneal neovascularization.

1.1 Angiogenesis: Physiology

Angiogenesis is a physiological process, defined as the growth of new blood vessels from preexisting ones. It differs from the process of vasculogenesis, which refers to the initial formation and development of new vessels from precursor stem cells, the angioblasts, during the early phases of embryogenesis. Angiogenesis takes place during embryonal and foetal development, but also in

the adult, in the context of wound healing, muscle growth, and growth of endometrium and placenta ^{4,7}.

1.1.1 The Vessel and the Endothelial Cell

The vascular system consists of arteries that transport oxygenated blood from the heart towards the periphery, and that divide into arterioles; from here the capillary network originates, a network of tiny vessels where the actual metabolic exchange with the tissues take place. The capillaries end in the venules that converge into veins, responsible for returning the deoxygenated blood to the heart and the pulmonary circulation. The vessel wall consists of a *tunica intima*, the internal lining of the vessel consisting of endothelial cells; an intermediate layer, or *tunica media*, where smooth muscle cells are located; and an external layer, *tunica adventitia*, with fibrous elastic tissue ⁸. The capillaries, on the contrary (Figure 1), have a wall consisting of only a monolayer of cells, the endothelial cells, with their basement membrane; the capillaries are wrapped in a particular type of cells called pericytes, that give structural support and contribute to metabolic regulation ⁹. Similarly to the brain, the capillaries in the anterior segment of the eye (for example in the ciliary processes), as well as in the retina form an almost impermeable barrier that protects the inner environment of the eye: the blood-aqueous barrier and the inner blood-retina barrier.

Fig.1: Schematic structure of a capillary vessel of the eye composed of endothelial cells, basement membrane, pericytes, and detailed illustration of the different adhesion structures between one endothelial cell and the other: tight junctions, adherence junctions, gap junctions, desmosomes (by Anthony Mukwaya).

The integrity and the barrier function of endothelia and epithelia depend on different adhesion structures (Figure 1): adherent junctions or *zonulae adherentes*, that are formed of VE-caderins and integrins, anchor one cell to another and connect the adhesion complex to the cytoskeleton; gap junctions, that form bridges between one cell and another, whose main component are connexins; desmosomes, that provide a strong structural network that binds cells together and anchors them to the cytoskeleton; and tight junctions, or *zonulae occludentes*. The tight junctions are complex adhesion systems between cells with “gatekeeper” functions, present only in vertebrates, and representing the molecular basis of barrier functions and of the strict regulated ion and nutrient exchange through mechanisms of transcytosis and paracellular transport, for example in the eye, the brain, the testis. The tight junctions consist of over 40 different proteins, like claudins, occludins¹⁰⁻¹². Tight junctions represent, for

this reason, the anatomical structure responsible for the blood-eye and blood retina barriers ¹³.

The vascular endothelial cell is a mesodermal cell covering the inside of the entire vascular system, stabilized by pericytes and by basement membrane ¹⁴, and that beyond structural functions present many different functions: barrier function, metabolic homeostasis, regulation of vessel tone, regulation of blood flow, clotting and hemostasis, inflammation, and angiogenesis. During inflammation and angiogenesis, the endothelial cells (ECs) express adhesion molecules, that cause leukostasis, leukocyte activation and extravasation, they secrete many cytokines, start the enzymatic degradation of the basal membrane, proliferate, differentiate in tip and stalk cells, and migrate ^{15,16}.

All tissues are composed of cells, and of an extracellular matrix, which fills the space outside the cells and is composed of glycoproteins, fibronectin, collagens, laminin and proteoglycans. The vascular extracellular matrix not only provides a scaffold for cellular support, but also participates actively in many processes such as angiogenesis and wound repair through complex interactions and signaling mechanisms ¹⁷.

1.2 Mediators and Triggers of Angiogenesis

The growth of new vessels by angiogenesis starts with an angiogenic stimulus such as hypoxia or inflammation. Similar to hypoxia, inflammation is considered to be a major trigger of angiogenesis ¹⁸, and its biochemical cascades are closely linked with angiogenic pathways ¹⁹. Inflammation triggers a cascade of mediators that leads to the destabilization of the vessel wall, the degradation of basal membrane and extracellular matrix, and the proliferation and migration of endothelial cells into the surrounding tissue, to form new vessels.

1.2.1 Vascular Endothelial Growth Factor (VEGF) and its Receptors

The main proangiogenic factor is vascular endothelial growth factor (VEGF), a growth factor produced both in response to hypoxia and to inflammation. Its main effects are the disruption of the vessel wall making it permeable through its effects on tight junctions, thus causing tissue edema, and the stimulation of endothelial cell growth into sprouts ²⁰.

The presence of a factor inducing the increased vascularization typical for tumors was postulated already in 1939 by Ide , but it was first in 1971 with Folkman that the idea of anti-angiogenesis as an anti-tumoral therapy was postulated ²¹. In 1983 the molecule was purified for the first time and called tumoral Vascular Permeability Factor (VPF) by Senger ²², and isolated and sequenced by Connolly in 1989 ²³; at the same time Ferrara sequenced a protein able to induce mitosis in endothelial cells and called it Vascular Endothelial Growth Factor ²⁴. cDNA cloning revealed that VPF and VEGF were the same molecule ^{25 26}.

VEGF belongs to a family of growth factors including VEGFA, B, C, D and Placental Growth Factor (PlGF), see Figure 2. VEGF C and D regulate lymphangiogenesis, while the main action of PlGF is in the placenta. VEGF-A, usually referred to as VEGF, is the most important one as it is the molecule responsible for angiogenesis and leakage, induces the growth of vascular endothelial cells, can induce angiogenesis in *in-vitro* models as well as in *in-vivo* models, is a survival factor for endothelial cells, and is a permeability factor that opens up tight junctions to induce leakage from vessels ^{27,28}. The VEGF-A gene is located on chromosome 6p21.3 and has eight exons and 7 introns; alternative exon splicing gives rise to four different isoforms, 121, 165, 189 and 206, 165 being the most common ^{29, 30,31 32}.

VEGF initiates its action by binding to specific receptors, located on the cell surface (Figure 2), but also to soluble unbound receptors that in the cornea maintain avascularity and transparency ³³. The VEGF receptors – VEGFR (Figure 2) - are a family of tyrosine-kinase receptors, characterized by having in common 7 immunoglobulin-like domains, a transmembrane domain, and an intracellular domain with tyrosinase activity able to induce a signaling cascade. The VEGFR family consists of three receptors, VEGFR1, 2, 3 and, in addition, neuropilin-1 (NRP-1) and NRP-2. VEGFR1 and 2 bind to VEGFA ³⁴, and VEGFR3 binds to VEGFC and D. VEGFR-2 is considered the most important one for the angiogenic effects, it binds to VEGF-A and have a potent tyrosinase activity starting a strong signaling cascade ^{35,36}. VEGFR1 binds to VEGF-B and to PlGF, but also to VEGF-A, but it has a much weaker downstream kinase activity and shows both pro-angiogenic and anti-angiogenic effects ³⁷. VEGFR-3 is specific for VEGF-C and VEGF-D and regulates lymphangiogenesis, but VEGF-C and VEGF-D have a weak affinity to VEGFR-2 as well ^{38 39}. NRPs can also play a role in angiogenesis forming complexes with other VEGFRs. VEGF receptors are found on many cells, mainly endothelial cells and on bone marrow-derived cells (monocytes), but also for example neural cells ⁴⁰.

Fig. 2. Schematic representation of Vascular Endothelial Growth Factor Receptors (VEGFR), their main ligands and their main functions (by Anthony Mukwaya).

VEGFA, which disrupts blood vessel walls and stimulates the growth of new vessels⁴¹, is a central mediator in both inflammation and angiogenesis. VEGFA is secreted by many cells, for example vascular endothelial cells, infiltrating leukocytes (myelomonocytes and neutrophils), corneal epithelium, retinal pigment epithelium, Müller cells, neural cells, and its expression is upregulated in the early phases of neovascularization^{42-44, 45, 46}. VEGF is also an inflammatory factor and it is released massively during the inflammatory cascade in response to different proinflammatory cytokines such as IL-6¹⁸.

1.2.2 Other Angiogenic Mediators

Many other substances have been shown to present angiogenic activity. Among these, several growth factors including Transforming Growth Factor- α (TGF α), TGF β , Keratocyte Growth Factor (KGF), Insulin-like Growth factor-1 (IGF1),

Fibroblast Growth Factor (FGF), Platelet derived Growth Factor (PDGF), neuropilins, angiopoietins, Tumor Necrosis factor (TNF). Most of these factors up-regulate VEGF. Moreover, VE-cadherin as well as the different matrix metalloproteinases (MMPs) induce angiogenesis^{47,48}.

1.3 Hypoxia

Cell metabolism is highly influenced by the availability of oxygen. Under hypoxic conditions, tissue suffers from a lack of oxygen and activates mechanisms aimed at compensating this deficiency. The blood flow is regulated in order to compensate for variation in oxygen: vascular endothelial cells secrete nitric oxide (NO), a vasodilator molecule able to increase blood flow to the hypoxic tissue⁴⁹. A prolonged low concentration of oxygen activates pathways that induce the transcription factor Hypoxia Inducible Factor-1 (HIF1). HIF1 is a heterodimer formed by HIF1 α and HIF1 β . In the presence of oxygen and iron, HIF1 α is stabilized by the binding of inhibiting molecules, or alternatively it is marked for degradation; in hypoxic conditions, however, it translocates to the nucleus where it binds to HIF1 β and becomes able to bind to Hypoxia Responsive Elements (HRE), specific areas of DNA expressed during hypoxia. HIF bound to HRE activates the transcription of factors related to angiogenesis, cell proliferation, metabolic adaptation and cell survival^{50,51}. These pathways lead also to the upregulation of VEGF⁵². VEGF then triggers the growth of new vessels aimed at restoring the oxygen balance. An example of this is the neovascularization taking place in the heart after a myocardial infarction, in the retina after a vein occlusion (RVO), in diabetic retinopathy (DR), and in retinopathy of prematurity (ROP). In the cornea, a relative hypoxia secondary to a chronic use of contact lenses may lead to limbal neovascularization⁵³.

1.4 Inflammation

An acute or chronic injury induces a complicated cascade of molecular and cellular reactions aimed at responding to and inactivating the peril. This complicated reaction is called inflammation and represents an indispensable defense mechanism toward a damage, infection or accumulation of a detrimental substance. Inflammation is characterized by five features classically described with the latin words *rubor* (redness), *calor* (heat) and *tumor* (swelling), all three related to hyperemia and increased vascular permeability ⁵⁴, *dolor* (pain) and *functio laesa* (impaired function). Many blood-derived cells and factors are needed in the tissue to orchestrate the inflammatory reaction, which is achieved both through vasodilation and leakage as well as the invasion of the tissue by new vessels. At the beginning of the inflammatory reaction a large number of circulating leukocytes and plasma proteins are attracted to the site ⁵⁵. These cells express inflammatory mediators like IL-1 α , IL-1 β and TNF ⁵⁶. As a response to TNF and angiopoietin-2 (Ang-2), an increased expression of adhesion molecules VCAM-1 and ICAM-1 on endothelial cells and leukocytes causes leukocytes to roll, stick and extravasate ^{57,58}. Many different plasma-derived factors such as complement proteins, antibodies, and acute phase proteins leak from the vessels into the tissue site of injury. The first (and most abundant) type of cells recruited during the inflammation process is neutrophils, followed by a smaller number of monocytes. The neutrophils produce inflammatory mediators that amplify the reaction functioning as chemoattractant to induce chemotaxis of more inflammatory cells, as well as vasodilation and increased vessel permeability. Among the cytokines and chemokines produced, a major role mediating vascular leakage and new vessel production is played by VEGF. The monocytes recruited to the extravascular tissue eventually mature into macrophages with phagocytotic activity in order to clean the tissue from debris of injured cells, infectious agents or foreign bodies. Other cells actively participating in the

process are mast cells, and more importantly the vascular endothelial cells. All the cells and factors recruited, after the acute inflammatory phase, eventually lead to resolution of inflammation and healing of the wound. The process described is an example of acute phase inflammation^{59 60}. If the cause of the inflammation is not removed, the process becomes prolonged and assumes different features, becoming a chronic inflammation. In the chronic phase, cell infiltration and cytokine production are less prominent, while tissue remodeling processes including angiogenesis take place. Many cytokines take part in the inflammatory reaction, but some key factors are TNF, IL-1 and IL-6. They are produced mostly by monocytic-dendritic cells, and they have a paracrine action, meaning that they act on cells and tissue near the site of their production.

A complex interplay between inflammatory and angiogenic factors, like the one between Ang-2 and TNF, or between IL-1 and VEGF, or IL-6 and VEGF, demonstrates the close relationships between inflammation and angiogenesis^{61, 62,63}.

1.4.1 Innate Immunity

The inflammatory reaction described above is a part of innate immunity.

Innate immunity is the body's first-line active defense, genetically the oldest part of the immune system, able to be activated very quickly, within minutes or hours. It is able to eliminate several kinds of pathogens, but also damaged cells. It activates the process of inflammation needed in order to clear pathogens or to start mechanisms of tissue repair. In order to respond promptly, it is activated in a rather aspecific manner, contrary to the specific response of the adaptive immune system, so that a quick defense can be in place. At the same time, it is able to provide danger signals that activate the adaptive immune system, which

needs time to prepare a more specific response through systems of gene rearrangement and lymphocyte recruitment eventually inducing cytotoxicity.

One of the earliest events of the innate immune system is the recognition of molecular signatures from foreign invading bodies (pathogen associated molecular patterns - PAMPs) and damaged cells (damage associated molecular patterns - DAMPs) that triggers signaling pathways and activates transcription factors. These pathways are mediated by surface cell receptors (Toll-like receptors) and cytosolic receptors (NOD-like receptors). One of the major transcription factors activated is nuclear factor kappa B (NF- κ B)^{64, 65, 66,67 68}.

1.4.2 Nuclear Factor kappa B (NF- κ B)

The transcription factor “nuclear factor (NF)- κ B” plays an essential role in innate immunity, inflammation, cell survival, cell differentiation and cell proliferation. Studies have reported the role of NF- κ B in angiogenesis through its regulation of the inflammatory response and VEGF expression^{69,70}. However, NF- κ B-dependent VEGF regulation is controversial and is reported to be cell- or tissue-specific⁷¹. The effect of NF- κ B activation appears to depend upon the stimulus, context of activation and cell type⁷². NF- κ B is located in the cytoplasm in its inactive dimeric form and is bound to the regulatory protein inhibitors of κ B (I κ B) family. Upon stimulation (for instance by inflammatory signals like TNF), I κ B kinase (IKK) complex phosphorylates the inhibitor I κ B subunit. This modification marks I κ B for degradation and enables nuclear translocation of the free NF- κ B^{73,74}. Nuclear NF- κ B binds to its target sequence and promotes transcription of a host of target genes, such as TNF, chemokine (C-C motif) ligand 2 (CCL-2; MCP-1) and chemokine C-X-C motif ligand 5 (CXCL5; ENA78). These factors can induce monocyte and neutrophil invasion

into tissue, respectively, and may in turn further activate NF- κ B signaling through their cell surface receptors. NF- κ B activation is controlled by the IKK complexes, that are formed by two kinases (IKK1 or IKK α and IKK2 or IKK β) and a regulatory subunit (IKK γ /NF- κ B Essential modifier - NEMO). Two NF- κ B pathways exist; the rapid canonical pathway turned on by proinflammatory stimuli associated with IKK2 and NEMO, and the slower IKK1-dependent non-canonical pathway related to lymphoid organogenesis⁷⁵. Inflammation-induced NF- κ B activation is associated with the canonical pathway resulting in I κ B α phosphorylation through IKK2⁷³.

1.4.3 The Complement System

An important component of natural immunity is a group of serum or surface proteins called complement system, which helps in the elimination of pathogens and infected cells facilitating the process of phagocytosis, or directly forming a membrane attack complex (MAC) able to kill foreign cells. The complement system may be activated by three cascades: in the so-called classical pathway, the first discovered, a serum protein called C1q activates the serine-proteases C1r and C1s, that start a cascade of proteolysis leading to the activation of C3; the alternative pathway is started by recognition of the microbial molecule lipopolysaccharide (LPS) directly by C3; finally, in the lectin pathway, a so called mannose-binding lectin (MBL) in the plasma recognizes microbial glycoproteins and glycolipids, and starts a proteolytic cascade. The common part of the complement pathway consists of the activation of C3 convertase, which cleaves C3 into C3a and C3b; C3b with other proteins constitutes C5 convertase, which cleaves C5 into its active form C5b; the latter binds to C6, C7, C8 and C9 to form the MAC, able to drill a hole in the membrane and thus provoke the lysis of the invading cell. C5a, on the other hand, has important inflammatory

functions, amplifying complement activation as well as recruiting inflammatory cells to the site. The other smaller fragments of the complement cascades have other functions like opsonization (facilitating phagocytosis of cells recognized by antibodies)^{59,60}.

The complement system is potentially dangerous for the integrity of the tissues, and is therefore strictly regulated by different mechanisms, like the presence of inhibitory proteins (C1 inhibitor, factor H, factor I, CD 59). The complement system is involved in numerous tissues and diseases. For example, in the eye, it is thought to be involved in the pathogenesis of AMD, since the risk of developing the disease is strongly linked with mutations in different complement-related loci^{76,77}.

1.4.4 Regulation of Inflammation and Innate Immune System

Many regulatory mechanisms modulate the immune response in order to limit and resolve the inflammation. Several cytokines have anti-inflammatory function, and many of them are specific for the innate immunity effectors. TGF- β is a typical anti-inflammatory cytokine, leading to the resolution of inflammation and healing, though promoting fibrosis⁷⁸. IL-10 inhibits macrophage activity. A natural antagonist of IL-1 is the endogenous IL1-receptor, an inactive molecule produced by macrophages that binds to IL1-receptor and blocks it. The inflammatory cascade itself leads to numerous negative regulatory signals, for example TLR-cascade induces the expression of SOCS, suppressors of cytokine signaling, that inhibit the JAK-STAT inflammatory signaling pathway⁵⁹. A number of other pathways play important regulatory roles in either promoting or suppressing inflammation.

1.5 Immune Privilege of the Eye

The eye is considered an immunologic privileged site, since important structures needed for vision are protected against potentially destructive immune response and inflammation. These features were recognized already in the beginning of the 20th century, when researchers noticed that grafts in the eye survived for a prolonged time. In the eye, there are several tissues and/or cells that are critical for vision and that cannot regenerate, for example neural cells and pigment epithelium in the retina and corneal endothelial cells. Inflammatory damage to these cells would be deleterious for vision. Similarly, the transparency and avascularity of the cornea must be preserved against vessel invasion and scarring. For these reasons, the eye is protected from the rest of the body by a series of barriers, and its milieu is anti-inflammatory and immunosuppressive through specific immunologic mechanisms: anterior chamber associated immunodeviation (ACAID) ⁷⁹, immunological ignorance, or tolerance to eye-derived antigens, prevalence of TGF- β ⁸⁰ and consequently IL-10, the presence of angiostatic and anti-inflammatory neuropeptides like somatostatin, and a suppression of IFN- γ and IL-12 ⁸¹. The cornea is protected on its surface by the tear film with its mucin layer, by the epithelial barriers, with its tight junctions, and by the limbal barrier. In corneal epithelium and stroma there's an abundance of endogenous anti-inflammatory and anti-angiogenic factors ^{82,83}.

1.6 The Angiogenic Process: Sprouting Angiogenesis

For the angiogenic process to occur, a cascade of events must take place involving the release of growth factors, degradation of the basement membrane, activation of tip cell, which leads the following sprout toward the growth factor

gradient, endothelial stalk cell proliferation, formation of solid sprouts of endothelial cells and connecting of sprouts to vascular loops ⁸⁴.

The endothelial cells interact with one another through a complex network of adhesion molecules linked to intracellular cytoskeleton: VE-caderins, but also Platelet EC adhesion molecule (PECAM-1) ⁸⁵. The cell-cell junction contributes to keeping the cells quiescent and the vessel stable (figure 1). The process starts with factors that induce overexpression of adhesion molecules on endothelial cells involved in leukocyte recruitment ⁵⁸. Many factors are produced by activated cells, among them VEGF that opens up tight junctions and destabilize the vessel wall ²⁸. The endothelial cells activated by growth factors as FGF and VEGF produce proteases, like matrix metalloproteinases (MMPs)⁸⁶ and collagenases, as well as urokinase-type plasminogen activator leading to active plasmin; the same growth factors also downregulate the physiological inhibitors of proteolytic enzymes, like Tissue Inhibitor of Metalloproteinases (TIMPs). The function of all these enzymes is to degrade the basement membrane and extracellular matrix ⁸⁷. The EC start to proliferate and migrate into the tissue. One EC differentiates into a tip cell, expressing VEGFR2, and is characterized by the presence of filopodia stretched into the tissue, that can sense and follow a gradient of chemoattractant, such as a growth factor (Figure 3) ^{88,89,90}. While the tip cell guides the growth of the new vessel sprout, the other cells differentiate into stalk cells, proliferate forming the trunk of the vessel, and shaping a lumen, connected to a pre-existing vessel (figure 3) ^{91,92}. Both tip and stalk cells are regulated through VEGF and VEGFR-2; the mechanism through which only the first cell acquires the phenotype of tip cell is through the signaling pathway of DLL4-NOTCH1, that in stalk cells gives rise to a cleavage product which acts as a transcription factor downregulating VEGFR2, 3 and NRP-1, and upregulating VEGFR1 and soluble VEGFR1 ^{93,94,95}. The lumen forms in a process influenced by many factors and by the blood flow itself, through apoptosis and through the

confluence of intracellular vacuoles that form in EC in response to integrin signaling pathway⁹⁶⁻⁹⁸.

Fig. 3. Schematic representation of sprouting angiogenesis. In response to a gradient of angiogenic trigger, the endothelial cells differentiate into tip cells, that guide the new sprout following a gradient of pro-angiogenic molecule with its filopodia, and stalk cells, forming the trunk and the lumen of the new vessel (by Anthony Mukwaya).

If the angiogenic stimulus is removed, remodeling, partial regression and stabilization of the newly formed vessels take place, with new pericyte coverage⁹⁹. This stabilization might render the vessels resistant to anti-angiogenic therapy¹⁰⁰. Different modes of capillary remodeling have been described, for instance endothelial cell apoptosis during the process of hyaloid vessel regression as well as pupillary membrane regression; or endothelial cell migration¹⁰¹⁻¹⁰⁶. Blood flow is also critical for the survival of the vessel¹⁰⁷. Capillary remodeling is linked to several different signaling cascades like Wnt¹⁰⁸, Angiopoietin and Tie1¹⁰⁹, as well as VEGF-B, which contribute to the survival of these stabilized capillary networks¹¹⁰.

This is the description of the most important mechanism of angiogenesis, sprouting angiogenesis. In addition, new vessels may be formed by

intussusceptive angiogenesis (or splitting angiogenesis), where a new wall grows inside an existing vessel, eventually dividing it into two new vessels^{111,112,113}, and looping angiogenesis, where vessel loops are mechanically dragged into the tissue^{114,115}.

1.7 Regulation of Angiogenesis and Angiogenic Balance

The process of angiogenesis is strictly regulated, both in time and space. A physiologic homeostasis is maintained by the so-called angiogenic balance, referring to a fine equilibrium between the factors inducing angiogenesis and the factors inhibiting it¹¹⁶. An angiogenic switch, however, tips the balance in favor of factors inducing angiogenesis, leading to activation of new blood vessel growth¹¹⁷. An uncontrolled vessel growth becomes pathologic and causes diseases. Some of the endogenous factors involved in the angiogenic balance are indicated in Table 1.

ANGIOGENIC FACTORS	ANGIOSTATIC FACTORS
VEGF family	VEGFR-1
Angiopoietin 1 / Tie 2	Soluble VEGFR-1
PDGF	VEGFR-3
TGF- β 1, TGF- β receptors,	Angiopoietin2
Endoglin	Trombospondin 1, 2
FGF	Angiostatin
HGF	Endostatin (collagen XVIII fragment)
MCP1	
Integrins	TIMP
VE-caderin	IL-4
PECAM (CD31)	IL-12
Plasminogen activators	IL-8
MMPs	IFN- α , β , γ
NOS-COX2	
HIF-1	

Table 1. Endogenous promoters and inhibitors of angiogenesis in the body.

1.8 Angiogenesis: Pathology

When pathologic conditions perturb the angiogenic balance tipping it in favour of neovascularization, an uncontrolled vessel growth may cause tissue damage. Pathologic angiogenesis or neovascularization is involved in more than 50 different diseases, including ocular, cardiovascular, inflammatory diseases, as well as cancer ⁴.

In tumours for example, the overproduction of angiogenic mediators, with both hypoxic and inflammatory stimuli, destabilizes the vessels and stimulates neovascularization making it possible for the tumour to keep on growing, and facilitating metastasis ^{118, 119, 120, 121, 122,123}.

Inflammation plays an important role in pathologic angiogenesis related to exudative macular degeneration, uveitis and infectious keratitis ¹²⁴.

Age-related Macular Degeneration (AMD) is a degenerative disease that represents the most prevalent cause of irreversible legal blindness in western world. Its wet form consists of a neovascular process originating from the choroidea (choroidal neovascularization, CNV), the pathogenesis of which is thought to be a chronic inflammatory process triggered by accumulation of toxic metabolic waste (drusen). Drusen forms due to a reduced efficiency of the retinal pigment epithelium (RPE), leading to a subsequent macrophage-derived VEGF-mediated neovascularization. Activation of the complement system plays an important role (SNPs related to the complement system are associated with increased risk for AMD) ^{125,126}.

In diabetes, the metabolic imbalance causes both a pro-inflammatory status (for example increased expression of adhesion molecules and pro-inflammatory cytokines), as well as microvascular impairment leading to hypoxia. The consequences are macular edema and retinal vessel proliferation, the two main mechanisms of damage in diabetic retinopathy, which represents the leading

cause of visual impairment in the population under 50 years of age in the western world ^{127,128, 129}.

In two further retinal neovascular diseases, retinal vein occlusions (RVO) and retinopathy of prematurity (ROP), hypoxia plays a major role. RVO is due to a thrombotic process in the retinal venous system with subsequent impairment of blood circulation, macular edema, and risk for proliferation; nevertheless, an inflammatory component is evident here also ¹³⁰. ROP is a vision threatening disease that can lead to retinal neovascularization, fibrous tissue formation and retinal detachment; this disease is caused by exposure of immature retinal vessels to high oxygen levels in neonatal intensive care units with consequent vasoconstriction and hypoxia ¹³¹.

1.8.1 Corneal Avascularity, Immune Privilege and Angiogenic Privilege

The cornea is the transparent window of the eye and is normally completely devoid of blood vessels in order to maintain its transparency. The cornea is characterized by an immune privilege, due to the barriers on the outer side (epithelium) and inner side (endothelium), and due to the particular anti-inflammatory milieu of the aqueous humor (anterior chamber associated immune-deviation, prevalence of antiinflammatory factors like TGF- β , see sections 1.5).

The cornea maintains a tightly regulated and delicate balance between pro-angiogenic and anti-angiogenic factors in order for it to stay avascular and transparent ¹³². The transparency and avascularity of the cornea are highly evolutionarily preserved features. This angiogenic privilege is made possible on one hand by passive mechanisms like corneal anatomy and barriers, on the other hand by active molecular mechanisms. The anatomy is characterized by densely

packed collagen fibrils, elastins and fibrins. More in detail, the cornea is formed by 5 different layers: epithelium, Bowman's layer, stroma, Descemet membrane and endothelium (Figure 4). The epithelium, densely innervated, and stratified in 5-7 layers of cells, is strongly attached to the underlying basement membrane with hemidesmosomes and adhesion molecules; in addition, the epithelial cells are connected to one another with tight junctions, an important feature for its barrier function¹³³; the outermost part of the epithelium is covered by a glycocalyx and moistened by the tear film. The Bowman's layer, a thin acellular layer composed of compacted collagen type I and V and proteoglycans, serves as a mechanical barrier to protect the stroma. The stroma is formed by collagen fibers type I and V disposed parallel to one another organized in densely packed lamellae; this particular arrangement allows transparency, and makes vessel ingrowth more difficult; other components are keratocytes immersed in an extracellular matrix. Posteriorly to the stroma is the Descemet membrane, an acellular layer formed of collagen type IV and VIII, and the corneal endothelium, formed by a monolayer of polygonal stable (terminal) cells that maintain a fluid and ion balance between the cornea and the anterior chamber, actively pumping water out of the cornea; they are connected with one another by desmosomes and *zonulae occludentes* (tight junctions), forming a barrier towards the aqueous humor in the anterior chamber¹³⁴⁻¹³⁶, see Figure 4.

Fig.4. The five layers of the cornea (by Per Lagman).

Besides tight junctions, the other passive protection is represented by the barrier function of limbus¹³⁷. The active protecting mechanisms are molecular strategies that allow angiostatic factors to prevail in physiologic conditions, and in this way to keep the vasculature of the eye stable¹³⁸. These mechanisms are: the presence of metalloproteinase inhibitors, soluble VEGF receptors³³, angiopoietin-1, TGF- β , vasoinhibin, and angiostatin^{138,139}, thrombospondin-1 and -2¹⁴⁰⁻¹⁴¹, endostatins¹⁴², and pigment epithelial derived factor (PEDF)¹⁴³. Some of them, like angiopoietin¹⁴⁴ and TGF- β ^{145,146} may have a dual effect, since they promote the stability of the vessel, but during angiogenesis promote capillary growth, depending on concentration. The angiogenic balance of the cornea is redundantly regulated by several different mechanisms, like the expression of VEGFR-3, where it can act as a decoy receptor for VEGF-C, or the presence of a soluble VEGFR-1, which binds and blocks the activity of VEGF-A to prevent angiogenesis^{33, 147,148}.

1.8.2 Corneal Neovascularization

The angiogenic balance can be disrupted by pathology such as injury, infection, or activation of an immune response, where inflammation plays a key role. Such triggers start the process of corneal neovascularization leading to tissue scarring, inflammation, lipid deposition, and corneal edema; in addition, the presence of vessels worsens the prognosis for corneal transplantation by facilitating immune-mediated rejection of the foreign transplanted tissue. All these alterations may result in a profound decline in vision. Globally, corneal blindness is the fourth largest cause of visual impairment after cataract, glaucoma and age-related macular degeneration¹⁴⁹. It is estimated that corneal neovascularization and scarring affects 1.4 million patients annually in the USA

alone. More frequently, though exact numbers are not available, corneal blindness due to neovessels and scarring occurs in developing countries where infections like trachoma are a major cause ¹⁵⁰. In the Western world, more frequent causes of corneal neovascularization are herpes simplex and herpes zoster viral infections. Other causes are immune disorders like pemphigoid, rosacea, and atopic keratokconjunctivitis, Stevens-Johnson syndrome, limbal stem cell deficiency secondary to chemical burns or aniridia, and hypoxia due to chronic use of contact lenses. Depending on the cause, the neovascularization may occur quickly, and be characterized by an inflammatory infiltrate, or may occur slowly with less inflammation ¹⁵¹.

An injury to corneal tissue causes necrosis of cells and the expression of DAMPs (damage signals) that elicit cascades of cytokine and chemokine production from the corneal epithelium, like for example IL-1 β and CCL2, in order to promote inflammation and to recruit immune cells ¹⁵²⁻¹⁵⁵. Epithelial damage also stimulates the production of angiogenic mediators like VEGF and TGF- β ¹⁵⁶. One of the earliest effects of this cytokine production is a vasodilation of the limbal arcade, and an increase in blood-derived immune cells and factors to the site of inflammation. The stroma is invaded by leukocytes ^{43,157-159}. An increased expression of adhesion molecules ICAM-1 and VCAM-1 ¹⁶⁰ causes leukocyte adhesion, activation and extravasation. Proteases are produced to degrade the extracellular matrix ^{161,162}. These cells, at a later stage could re-activate vessel growth upon cessation of treatment ¹⁶³⁻¹⁶⁵.

The edema caused during the inflammatory process may also facilitate neovascularization. Neutrophils and monocytes secrete chemotactic factors that amplify the reaction. Many of the effects of the inflammatory cytokines produced are mediated by NF-kB. New blood vessel growth is characteristically driven by a gradient of VEGF. The newly formed sprouts are immature and leaky. Stabilization of the newly formed vessels requires recruitment of

structural support cells, the mural cells: pericytes for the capillaries and smooth muscle cells for arteries and veins. This process is mediated by PDGF signaling as well as Ang-1 and its receptor Tie2^{166,167}.

Resolving inflammation can be crucial for managing corneal neovascularization; however, the various activated inflammatory pathways and their temporal regulation are still poorly understood^{168,169}.

1.9 Wound Healing and Angiogenesis

Wound healing requires functional angiogenesis that provides nutrients and oxygen to support cell growth, tissue repair, granulation matrix formation, as well as to clear the associated debris¹⁷⁰. Wound repair usually requires three to fourteen days to be completed and starts after hemostasis, which consists of platelet aggregation, thromboplastin production, and vasoconstriction. The process of wound healing starts with the inflammatory phase, characterized by chemotaxis of neutrophils and macrophages to the site of injury, in order to remove harmful triggers such as bacteria or remnants of injured cells, and in order to produce inflammatory and angiogenic mediators as well as growth factors. The second phase is the proliferative phase: fibroblasts produce extracellular matrix while new vessels grow in the granulation tissue; at the same time, epithelial cells at the edge of the wound proliferate and migrate towards the center in order to cover the breach. Lastly, in the remodeling phase, the collagen matrix as well as the newly formed vessels are reorganized and stabilized, new collagen is formed, and the fibroblasts become myofibroblasts giving rise to scar contraction and strengthening of the tissue¹⁷¹.

1.9.1 Wound Healing Process in the Cornea

The success rate of ophthalmic procedures such as glaucoma filtration surgery, cataract extraction and corneal transplantation is greatly affected by post-operative wound healing in the cornea. Corneal wound healing is a multistep process where the stromal extracellular matrix proteins and growth factors excreted by the epithelium send the first signals of cell injury, followed by re-shaping of the epithelial cells. Early wound closure appears to be independent from limbal activity as the epithelium in the center of the cornea migrates to close the gap^{172,173}. After initial closure of the wound, however, the limbus is activated to initiate the delivery of transient amplifying cells, which differentiate into basal epithelial cells. Basal epithelial cells start accumulating at the wound site and eventually migrate upwards to form a thicker, stratified corneal epithelium^{174,175}.

In order to migrate and proliferate, epithelial cells need to uncouple their connections with one another. Among these connections, crucial are gap junction that form channels between adjacent cells. The gap junction alpha 1 (GJA1) gene encoding human connexin 43 (Cx43) was previously reported to be differentially expressed during wound healing¹⁷⁶⁻¹⁸⁰; alteration in the expression of Cx43 is associated with heart disease¹⁸¹ and cancer^{182,183}. The basal layer of the corneal epithelium is known to exhibit Cx43 positivity, and the expression of Cx43 is downregulated on the migrating edges of open wounds^{177,184-186}. A transient downregulation of Cx43 is important in the initiation of the epithelial cell migration process in the early wound healing stages. This facilitates uncoupling of adjacent connections between cells, which is essential for epithelial cell migration to close the wound gap. Pharmacological and genetic targeting of Cx43, which can transiently block gap junctions' hemichannels, may accelerate wound healing of vascular and avascular tissue^{179,187-190},^{187,190}.

1.10 Imaging of Angiogenesis

Both in clinics and research, it is crucial to have adequate imaging tools in order to assess a disease process, and follow-up therapy. In medical science in general and in ophthalmology in particular, major advances in imaging techniques provide new tools for clinics and research. For ophthalmic neovascular disease we can image the presence of neovessels, as well as their effects like edema and scarring, for example in retina with OCT (optical coherence tomography) and angiography, where technical innovation provides *in vivo* images with a resolution similar to histology. In cornea on the other hand, neovessels are easily detected by slit lamp examination without the use of complicated imaging systems. Slit lamp is in use since 1911, and is the standard examination to quantify the degree of visible neovascularization as a means to grade the extent of pathology and evaluate the efficacy of anti-angiogenic treatment. However, damage to the tissue can be inflicted before the first signs of vessel ingrowth and even in the absence of vessels, by means of inflammatory cell invasion. Inflammatory cells such as neutrophils and macrophages, indeed, are important sources of pro-angiogenic cytokines, which precede vessel invasion and at a later stage could re-activate vessel growth upon cessation of treatment. A more complete assessment of anti-angiogenic therapy would therefore include quantification of the suppressive effect on neovessel growth and on the dynamics of tissue inflammation¹⁹¹⁻¹⁹⁴. Current methods to assess the efficacy of anti-angiogenic treatments have overwhelmingly focused on examination with slit lamp and photography¹⁹⁵⁻¹⁹⁹. It is possible, however, to detect early signs of inflammation by *in vivo* confocal microscopy (IVCM)²⁰⁰. IVCM is based on scanning confocal microscopy technique that increases optical resolution and contrast blocking all out of focus light through a pinhole (Figure 5)²⁰¹. Confocality improves the resolution, but allows only small areas to be analyzed at the same time; for that reason the machine uses a scanning technique

in order to build a picture. Several different microscopes are available, but laser scanning confocal microscopy uses a coherent light source that has the advantage of providing better contrast and resolution (achieving a resolution of 2 μm laterally and 4 axially). This technique provides quick and reliable visualization of all microstructures of the cornea: epithelium, tear film, keratocytes, nerves, endothelium ²⁰². Advantages like the visualization of cellular structures and the possibility to perform real time sequential in vivo observations have granted a role of IVCM in clinics for corneal pathologies as acanthamoeba and fungal infections, corneal dystrophies and degenerations, as well as assessment of nerve status ²⁰³⁻²⁰⁶. Our research group was the first to use IVCM to assess angiogenesis.

Fig.5. Schematic illustration showing the optical principles of confocal microscopy (by Per Lagman).

1.11 Therapy of Pathologic Angiogenesis

1.11.1 Anti-VEGF Treatment

The first idea of blocking the angiogenic process for therapeutic aims was by Folkman in relation to cancer ²⁰⁷. Since VEGF is responsible for disrupting blood vessel walls and stimulating the growth of new vessels and represents a central mediator in both inflammation and angiogenesis, it is logical that blockade of VEGF can have therapeutic implications in many diseases. Chimeric monoclonal antibodies (bevacizumab, ranibizumab) and decoy receptors (aflibercept) have indeed been developed towards VEGF and have been widely used therapeutically: anti-VEGF therapy has revolutionized the prognosis of wet AMD ²⁰⁸⁻²¹⁰, and is an established treatment modality for different retinal diseases such as diabetic macular edema ²¹¹ and RVO ²¹², although repeated treatment (typically monthly intravitreal injections) are required in order to sustain angiogenic suppression. Moreover, therapy fails in at least 20% of cases ^{208,209}.

In oncology, anti-VEGF is used to augment treatment of many types of tumors, but its value is limited by escape mechanisms and resistance to anti-VEGF treatment that often develops over time ^{213-215 216,217}.

In recent years, anti-VEGF has been used to treat corneal neovascularization as well ^{169,218}. While this more targeted treatment avoids the side-effects of steroids, experimental and clinical studies have shown only a limited reduction in corneal neovessels. Using locally-applied anti-VEGF by topical ¹⁹⁷, subconjunctival ²¹⁹, intrastromal ²²⁰, or intraocular route ²²¹, neovessel reduction in the range of 15-20 % in experimental studies ²²² and of 36-61% in clinical studies ^{197,219,223} has been reported. Anti-VEGF may also have a shorter therapeutic window compared to steroids ¹⁵⁹. The reason for this limited and variable efficacy, however, remains unclear, but could be due to multiple redundant angiogenesis

pathways that are activated upon suppression of VEGF, and in the cornea, the lack of addressing inflammatory pathways.

1.11.2 Corticosteroid Treatment

Glucocorticoids, often called with the more generic name of corticosteroids, or steroids, are broadly used in medicine because of their strong anti-inflammatory effect.

Corneal neovascularization has traditionally been treated with topical steroids in the active phase, whereas in a later phase only surgical procedures with often poor prognosis, if any therapy at all, are possible^{224,225}. Steroids are known from clinical practice to be effective, nevertheless their sustained use can have frequent and serious side effects, including secondary glaucoma, corneal thinning and perforation, cataract, and herpes infection^{225, 226, 227, 228}.

In some retinal diseases steroids are used since they block the cascade of arachidonic acid, and thus inhibit even the production of VEGF. For that reason, they are effective on macular edema due to diabetes and RVO²²⁹.

Due to the risks associated with the use of immunosuppressive steroids on the one hand, and the limited efficacy of anti-VEGF treatment in suppressing angiogenesis on the other, alternative treatments are sought.

1.11.3 Other Treatments for Corneal Neovascularization

Other possible treatments for corneal neovascularization are non-steroidal anti-inflammatory drugs, laser photocoagulation²³⁰, fine needle diathermy²³¹, and photodynamic therapy^{232,225}. These treatments are, however, of variable efficacy. Aganirsen (GS-101®) is a DNA antisense oligonucleotide targeting

insulin receptor substrate-1 (IRS-1), that has shown promising results in inhibiting keratitis-induced corneal neovascularization^{233,234}.

1.12 Research in Angiogenesis: Models of Pathologic Angiogenesis

Relevant models of angiogenesis are crucial to gain insight into the angiogenic process and to test the effect of new experimental compounds on vascular endothelial cells and the process itself. The models can be broadly classified into *in vitro*, *ex vivo* and *in vivo* models.

In vitro models are based primarily on endothelial cell cultures, like human umbilical vein endothelial cells (HUVEC), that can be cultured alone or co-cultured with other cells like fibroblasts and pericytes on collagen for two-dimensional studies or matrigel for three-dimensional studies^{235,236}. *In vitro* models include endothelial cell proliferation assays, endothelial cell migration assays (transfilter assay and others), endothelial cell differentiation assay (tubule formation on matrigel), and the endothelial cell-mural cell co-culture assay²³⁷. In this thesis, also human corneal epithelial cell (HCEC) cultures are used, to assess wound healing. These models are very practical and versatile and give the possibility to manipulate the medium in which the cells are cultured, but have, however, severe limitations in the lack of the complex physiologic milieu characterized by yet not fully understood biochemical interactions and signaling.

Ex vivo models are based on organ cultures, for example rat aorta ring assay²³⁸, or vena cava explant. An *ex vivo* model used in this thesis is a human corneal culture model to assess wound healing and inflammation. In these models, the presence of whole tissues with different kind of cells interacting together mimic better the physiologic condition, thus lacking important elements like blood flow and inflammatory signaling²³⁹.

In vivo models are thus essential. Many different *in vivo* models have been described, such as chicken embryo chorioallantoic membrane assay, cheap, effective and widely used^{240,241}; mouse hindbrain model²⁴², rabbit ear chamber²⁴³, dorsal subcutaneous air sac²⁴⁴.

Alternatively, zebrafish models are convenient for drug testing and angiogenesis studies due to optical transparency, rapid embryogenesis, and an angiogenic process similar to other vertebrates^{245,246}. Zebrafish embryos are used for developmental studies of vasculogenesis; tail fin model and retinal hypoxia model are used to study tumor angiogenesis and retinal neovascularization^{247,248,249, 250}. Mouse retina has been widely used during the last years with laser induced CNV as a model for wet AMD, and oxygen induced retinopathy (OIR) as a model for ROP^{251, 252}.

The most important angiogenesis models, from where much of our knowledge in the subject derives, are corneal neovascularization models. Corneal angiogenesis models allow a direct visualization of neovessels, given the transparency and avascularity of normal corneal tissue. A variety of corneal angiogenesis assays have been proposed, using rabbit, guinea pigs, mice, and rats. The neovascular response is triggered by active substances introduced into the stroma via pellet insertion into a micropocket^{253,254}, or intrastromal injection, by chemical cauterization, mechanical scraping, surgical grafts, or intracorneal sutures; medications can easily be given locally as eye drops or by sub-conjunctival injection¹⁹⁵. Corneal models require complicated surgical procedures and are expensive; another limitation is represented by the peculiar features of corneal tissue that differentiate it from other tissues, such as immunologic and angiogenic privilege. For these reasons, the different models and assays of angiogenesis should be considered complementary to one another.

The vascular response in corneal models is usually studied *ex vivo* by perfusion of the cornea with dye followed by fluorescent imaging or by immunochemical

staining followed by confocal microscopy. Corneal angiogenesis is typically characterized by a measurement of the vascularized area (expressed as percentage of the whole cornea), vessel length, vascular density, and capillary diameter^{255,256}. One problem of most proposed models and assays is that the complex process of angiogenesis is usually frozen in different stages, and followed over time only in different individuals, with risk of inter-individual variations affecting the results, but also requiring the use of many animals.

1.12.1 Suture-induced Inflammatory Corneal Neovascularization (Papers I-II-III-IV)

In this thesis, a model of inflammatory neovascularization in the rat cornea was used as previously described by our group (Figure 6)²⁵⁷. The model is characterized by the advantage of allowing a direct observation and quantification of the neovascular response, owing to the transparency and avascularity of the cornea. The rat cornea is large enough to allow good possibilities of *in-vivo* imaging. In this model, the growth of neovessels is triggered by intrastromal suturing. This procedure is done in a standardized and repeatable manner. Left eyes are left untouched in sutured animals. One or two sutures, depending on the study, are placed 1.5 mm from the temporal limbus (Figure 6, 7). This distance derives from the previous studies of our research group that established the model, as too short a distance induces a rapid and intense neovascular response where single vessels are difficult to see and quantify, while too long a distance induces a very light and slow response, if any. The response is then studied with different imaging techniques *in vivo* (see next Section 1.12.2), as well as histology and/or immunohistochemistry (IHC), followed by molecular and gene expression analysis of corneal tissue samples (Figure 7).

The intensity and extension of the neovascular response in this model may be modulated by different parameters such as the number of sutures, pattern and depth of suture placement, suture size and material, and the distance of the sutures from the limbus: the closer the sutures are to the limbus, the faster the neovascularization response; the greater the number of sutures spread out across the cornea, the larger the neovascularized area. This model is relevant also because it mimicks corneal neovascularization due to corneal sutures after corneal transplantation. The process is then followed longitudinally, with or without different treatments. In this thesis, three active treatments given topically, and one systemic treatment, have been tested: topical rat specific anti-Vegf, topical dexamethasone, topical Gap 27, and systemic IMD0354. Control substances were topical IgG, scGap27, CMC (vehicle).

Fig.6. *The suture model of inflammatory corneal angiogenesis used in this thesis. A: appearance of a naïve cornea (the visible vessels are iris vessels behind). B: distance measurement from limbus (1,5 mm) with a caliper. C: two surgical intra-stromal 10-0 nylon sutures are placed (anesthesia, analgesia and antibiotics are used). D: corneas are examined at various time-points. At day 4, capillary sprouts extend halfway to the sutures; the suture is reached by day 7 (by Anthony Mukwaya).*

Fig.7. Schematic representation of study design of Paper I and II. A similar study design was also used for the in vivo experiments on the same model of Paper III and IV, alongside with other models (zebrafish embryo, aortic ring assay, human cornea culture model, cell cultures with HUVECs and HCECs). The animals (rats) receive one (Paper I) or 2 sutures (Papers II, III, IV) 1.5 mm from temporal limbus at day 0. The topical treatments (dexamethasone, rat-specific anti-Vegf, Gap 27, IgG, scGap27) are given QID (4 times a day), alternatively systemic treatment with IMD0354 or CMC. Corneas are studied in vivo at various time-points with slit lamp photography and IVCM. In Paper I and Paper IV the neovascularization process was followed until day 7 in order to quantify the vessels. In Papers II and III focus was given to the early time-points until 48 hours. At the experimental endpoint, tissue is harvested for histologic, molecular and genetic analysis with HE, immunofluorescence, microarray, qPCR, Western blot (by Pierfrancesco Mirabelli).

1.12.2 Imaging of Angiogenesis by *In Vivo* Confocal Microscopy (IVCM)

In this thesis, we used laser scanning in vivo confocal microscopy (IVCM), with an instrument clinically approved for use in imaging the human eye (Heidelberg Retinal Tomograph 3 with Rostock Corneal Module, Heidelberg Engineering). This instrument allows microscopic analysis of the corneal tissue *in vivo*, with the advantage of being able to follow the process longitudinally, gaining valuable information in single corneas, as well as limiting the number of animals needed for the studies. The microscope was equipped with a 63x/0.95 NA water-immersion objective (Zeiss, Oberkochen, Germany), that provides an *en face* view of 400 μm x 400 μm corneal area at a selectable corneal depth²⁵⁸. IVCM has been shown to be a valuable tool for imaging of various cells in transparent tissue, non-invasively, in a time-lapse manner, without the need for cell labeling (Figure 8)^{200,258}.

Fig.8. IVCM pictures of rat cornea with infiltrating inflammatory cells (left picture and right picture, short arrows) and vessel sprouts growing into the stroma (right picture, long arrows). Pictures by our study group.

2 AIM OF THE WORK AND RESEARCH QUESTIONS

Available anti-angiogenic treatments for neovascular diseases are not ideal, with anti-VEGF agents showing limited efficacy and requiring long-term administration of therapy, and immunosuppressive steroids potentially inducing serious side-effects. For these reasons, new treatments are sought. The studies presented in this thesis are conceived as a contribution towards the quest for more effective treatments for corneal neovascularization with fewer side-effects. The main hypothesis is that we need to address not only the angiogenic cascades but also the inflammatory component of the process. The more specific the treatment (instead of the broadness of action of steroids/glucocorticoids) the less likely it is for the treatment to induce side-effects; at the same time VEGF as the only target seems insufficient since redundancy in the angiogenic process provides many rescue pathways able to bypass a VEGF-blockade. Specifically, mimicking the steroids, that is addressing the inflammatory component of the angiogenic response as steroids do, but in a more targeted manner than steroids, may avoid side effects while potentially improving the efficacy. In order to achieve such a treatment, we need first to study more in depth the details of the angiogenic process and to understand how current therapies influence it. Many of the underlying mechanisms are still unclear.

Within this context, the main research questions motivating the studies presented in this thesis are outlined below.

2.1 Early Markers of Inflammatory Angiogenesis

Research Question 1: What are the early markers of inflammatory angiogenesis? (Paper I, II, III).

The first aim of the research is to study in detail and describe the early pre-sprouting changes of the neovascular response, and in this way identify early markers of inflammatory angiogenesis.

In the early phase of the pathologic process, cytokines and chemokines drive leukotaxis, *id est* migration of leukocytes, mainly neutrophils and macrophages, from capillaries and surrounding tissue; these leukocytes amplify the response through further cytokine production. Many inflammatory factors are described in ocular angiogenesis, for example TNF, IL-6, IL-1, IL-8, and IL-1 β . This early inflammation and damage is often invisible upon clinical examination, but it most likely represents the time window in which most of gene activation and inflammatory factor production takes place; all these changes produce in a later phase the visible signs of dilated preexisting capillaries and sprouting angiogenesis. Our group has previously showed that it is possible to detect early signs of inflammation by in vivo confocal microscopy (IVCM) ²⁰⁰. With this thesis, we aim to study more in detail this early pre-sprouting phase, believing that it is most likely the phase in which to intervene therapeutically in order to arrest or modulate the neovascular process for best efficacy. Can we prevent neovascularization blocking the secretion of inflammatory pro-angiogenic factors in the early phase?

2.2 Modulation of Neovascular Response by Anti-angiogenic and Anti-inflammatory Treatment

Research Question 2: How do anti-angiogenic and anti-inflammatory treatments (VEGF-blockade, glucocorticoids, NF- κ B inhibition) modulate the early phase of corneal neovascularization? (Paper I, II, III).

The second aim of the research is to study how available anti-inflammatory and anti-angiogenic treatments modulate the early phases of the neovascular response. Here it was sought to compare the differences in anti-angiogenic effect of the two available treatments, anti-Vegf and steroids, given topically, relative to a control substance. This comparison was done in the early phase of the neovascular response in an inflammatory corneal neovascularization model, analyzing the cornea corneal tissue at the cellular, tissue, and gene expression levels. Our hypothesis was that dexamethasone has a broader and more intense effect than anti-VEGF on angiogenesis inhibition, because it targets both VEGF-dependent and VEGF-independent pathways. We wanted to investigate which biological processes are modulated by these treatments, in order to find on the one hand which processes are responsible for the difference in effect between the treatments; on the other hand which processes are necessary for normal biological functions, and may become responsible for side-effects. The knowledge of how these processes are influenced may contribute to avoiding undesired effects, and to finding more effective treatments.

2.3 Characterization of VEGF-independent Pathways, and their Therapeutic Modulation.

Research question 3: Which are the most important VEGF-independent pathways in corneal neovascularization, potentially responsible for the limited efficacy of therapy? (Paper I, II, III).

Here the aim was to identify the presence of other pro-angiogenic cytokines not targeted by current anti-angiogenic treatments, especially those related to inflammation or produced by inflammatory cells. Such cytokines might represent future targets for more effective and more selective treatments with fewer side-effects. VEGF-independent pathways are still poorly characterized in many angiogenesis models but are believed to be responsible for escape mechanisms leading to disease relapse or treatment resistance towards anti-VEGF. During the early phase of inflammation, we performed genome-wide microarray analysis in anti-Vegf and dexamethasone treated animals, in order to investigate systematically the differential effect of these treatments at the gene expression and pathway activation levels. This knowledge could be used to block specific factors or pathways in order to achieve more targeted and effective therapies.

2.4 Efficacy, Specificity and Side-effects: Anti-angiogenic Effects of Selective Inhibition of NF- κ B Pathways

Research question 4: Does specific inhibition of the inflammatory cascade lead to improved efficacy in targeting inflammatory corneal neovascularization? (Paper III).

Resolving inflammation can be crucial for managing corneal neovascularization; however, the various activated inflammatory pathways and their temporal regulation are still poorly understood. Despite the identification of a number of important inflammatory cytokines and factors involved in pathologic corneal inflammation and angiogenesis, it is unclear which pathways are best to target in order to achieve effective dampening of inflammation and angiogenic response. As a means to avoid broad pharmacological targeting, as it is the case with glucocorticoids, but at the same time as a means to specifically suppress the downstream chemokines of the inflammatory cascade, we studied the potential anti-inflammatory and anti-angiogenic properties of a small-molecule, IMD0354 that targets NF- κ B signaling pathway through selective inhibition of one of its subunits, IKK2.

2.5 Wound Healing and the Endogenous Modulation of Inflammation and Angiogenesis in Cornea

Research question 5: Does acceleration of wound healing inhibit inflammation and angiogenesis? (Paper IV).

In physiology, the angiogenic response clears up the inflammation, and plays an indispensable role in wound healing. Inflammation, angiogenesis and wound healing are thus closely interconnected processes, finely regulated in time and space. We hypothesize an endogenous switch that can be triggered to activate and deactivate the process. The aim of this study is to investigate if accelerating wound healing might switch down the angiogenic process and thus avoid corneal neovascularization. For this reason, we studied the effects of blockade of Connexin 43, the gap junction $\alpha 1$, through the mimetic peptide Gap27, on corneal wound healing and on inflammation. Corneal wound healing is a multistep process where the stromal extracellular matrix proteins and growth factors secreted by the epithelium send the signals of cell injury, followed by reshaping and migration of the epithelial cells to close the breach; this migration need uncoupling of intercellular connection and thus a transient downregulation of Cx43. The effect of Gap27 on corneal wound healing, inflammation and neovascularization was tested on human corneal epithelium *in vitro*, on whole human corneas *ex vivo* and in rat corneas *in vivo*.

3 MAIN FINDINGS

Early Markers of Inflammatory Angiogenesis (Paper I, II, III)

Already 5 hours after suturing, a rich cellular infiltration was observed in all corneas by IVCN, and confirmed by immunofluorescence, with a clear wavefront between invaded and un-invaded corneal stroma; these cells are myeloid cells expressing CD11b+ and CD45 (Papers I, II, III). The inflammatory infiltration was confirmed also by the cellular infiltration in the normally acellular aqueous humor (Paper II, III). The in vivo morphology of macrophages is known from an earlier study^{191,258}, and was in addition confirmed by immunostaining with the mature macrophage marker Ki-M2R. Mature macrophages were observed in vivo to accumulate at the suture site by day 2.

Prior to sprout development (which occurred at day 3-4), another evident feature in all sutured corneas was limbal vessel dilation, observed at both 5 and 48 hours, and assessed by IVCN imaging; this finding was consistent in all studies (Paper I, II, III, IV).

Modulation of Neovascular Response by Anti-angiogenic and Anti-inflammatory Treatments (Paper I, II, III).

In vivo measurement of vessel dilation revealed that early-phase limbal vessel dilation at 5h was inhibited in both dexamethasone and rat specific anti-Vegf treatment groups in comparison to controls (Paper I); at 48 h, limbal vessel dilation was effectively suppressed by dexamethasone, but not by anti-Vegf (Paper I, Figure 9, Paper II). Similarly, selective NF-κB blockade suppressed vasodilation at 5 h, 48 h and 96 h (Paper III).

Fig.9. Representative IVCN pictures of limbal vessels in IgG, anti-Vegf and dexamethasone treated corneas (left); quantification of limbal vessel diameter in the three groups (Paper I).

No treatments blocked cell infiltration (Figure 10). Despite blocking vasodilation, anti-inflammatory treatments did not suppress inflammatory cell infiltration into the cornea. CD45-positive inflammatory leukocytes invaded all sutured corneas regardless of treatment (Paper I, II, III). Quantitative analysis on IVCN images demonstrated no significant difference in inflammatory cell infiltration at 5h and 48h between dexamethasone and anti-Vegf (Paper I, II). Both treatments showed only a delay of the early migration of myeloid cells at 5 h compared to controls (Paper I). NF- κ B inhibition reduces infiltration at 5, 48, 96 h relative to controls.

Fig.10. Representative IVCN pictures of infiltrating inflammatory cells in corneal stroma in IgG, anti-Vegf and dexamethasone treated corneas (Paper I).

Anti-Vegf and dexamethasone, given topically, both significantly reduced the number of cells in the aqueous humor, but similar to the corneal stroma there was no significant difference between the treatment groups (Paper II). Reduced aqueous infiltration was also observed in NF- κ B inhibition (Paper III).

Macrophage quantification at the suture site revealed that dexamethasone treatment significantly reduced the recruitment of macrophages at day 2 in comparison to anti-Vegf treatment and controls. By day 7, the number of macrophages had increased in all groups, but the total number of macrophages in anti-Vegf and dexamethasone-treated corneas was still significantly reduced in comparison to controls (Paper I).

Photographic analysis *in vivo* revealed that dexamethasone treatment significantly suppressed corneal neovascularization at day 7, with a reduction of total vessel length by 95.3%, and vessel invasion area by 93.1% relative to controls; while anti-Vegf was significantly weaker than dexamethasone ($P < 0.05$) with a reduction of vessel length by 14.4% and invasion area by 13.8% (Figure 11, Paper I).

Fig.11. Left: Representative pictures of corneas treated topically with IgG (controls), rat-specific anti-Vegf, dexamethasone. Right: quantification of neovascularized area (Paper I).

NF- κ B blockade decreases neovessel density in the cornea at 96 h (in a semi-quantitative scale 0-3, treated corneas were graded 2, that is one step milder neovascularization than controls, graded as 3); however, neovascularization at 96 h was not prevented (Figure 12, Paper III).

Fig. 12. Semi-quantitative assessment of neovascularization grade (scale 0-3) at 96 h in corneas treated with active IMD0354 inhibiting NF- κ B, and control substance CMC (Paper III).

Characterization of VEGF-independent Pathways, and their Therapeutic Modulation (Paper I, II, III)

In paper I, selected major angiogenic, inflammatory and macrophage-related factors were analyzed by qRT-PCR at 24 h and 7 d, but no significant difference could be detected between dexamethasone and anti-Vegf: *Vegfa*, *Il-6*, *Fgf-2* and *Tnf* were down-regulated by both dexamethasone and anti-Vegf. No modification of *Ccl2* expression could be detected at 24 h, but a down-regulation became evident by day 7. No modification of the expression of *Ccl3* and *Cxcl2* could be detected at either 24h or at day 7. The expression of *Vegfa* in the corneal stroma was partially suppressed by both anti-Vegf and dexamethasone relative to IgG (Western blot and immunofluorescence).

To investigate factors suppressed by dexamethasone but not by anti-Vegf treatment, in paper II a genome-wide expression analysis using RNA microarray was performed 48h after suture placement in IgG (control substance), dexamethasone, and anti-Vegf treated groups. Differentially expressed genes (DEGs) in key biological processes were preferentially suppressed in dexamethasone versus anti-Vegf treatment. More in detail, a total of 2048, 1659

and 2170 genes were differentially expressed in IgG, dexamethasone and anti-Vegf treated groups, respectively, relative to naïve (control) corneas; in almost all key biological processes regulating the inflammatory response, and angiogenesis, dexamethasone had fewer DEGs represented than in IgG or anti-Vegf groups. Only marginal differences were noted between IgG and anti-Vegf. Biological process enrichment analysis and pathway analysis showed that many biological processes (cell differentiation, regulation of apoptosis, cell adhesion, immune response, and inflammatory response, see Figure 13) and pathways (PI3K-Akt signaling, focal adhesion, and NF-κB signaling) were perturbed by steroid treatment.

Fig.13. Biological process analysis showing the processes with highest number of DEGs (differentially expressed genes) for Dexamethasone, anti-Vegf, and the control substance IgG (Paper II).

Dexamethasone suppressed several inflammatory factors, and enhanced specific complement factors, relative to anti-Vegf: *C3*, *Ctgf*, *C1s*, *Cxcl5*, *Reg3g* and *Ccl2* were the most differentially regulated genes, with *C3*, *Ctgf* and *C1s* upregulated by dexamethasone, while *Cxcl5*, *Reg3g* and *Ccl2* downregulated by dexamethasone, both relative to anti-Vegf (Table 2). In addition, dexamethasone suppressed strongly the expression of *Serpinb* and *Cfi*, and upregulated *C2* and

C3. The analysis was confirmed by immunofluorescence (Figure 14), Western blot and by quantification of fold change difference. Because of the involvement of multiple complement genes, microarray data for genes of the complement cascade were examined in detail. Nine complement genes were differentially regulated by dexamethasone relative to anti-Vegf, all of these being confined to the early complement cascade (*C1 – C3*), in addition to *Cfi*. The expression profile for *C3*, *C1s* and *Vegfa* in all the treatment groups was verified by qPCR, confirming the trend of gene expression as shown by microarray, IHC and Western blot analysis (Figure 14).

Gene ID	FC Dex-Vegf	Gene ID	FC Dex-Vegf
Ccl2	-38,76 (P<0,01)	C3	16,56 (P<0,01)
Reg3g	-15,53 (P<0,01)	Ctgf	4,26 (P<0,01)
Cxcl5	-7,64 (P<0,01)	C1s	4,11 (P<0,01)
Dowregulated by dex		Upregulated by dex	

Table 2. List of the top three genes downregulated respectively up-regulated by the treatment with dexamethasone in our model of inflammatory neovascularization in cornea, relative to anti-Vegf treatment. Fold change difference between dexamethasone and anti-Vegf is given, as well as p-value (Paper II).

Fig. 14. Immunofluorescence of naïve corneas, and of corneas treated with IgG, anti-Vegf and dexamethasone, confirming the modulation of gene expression of C3, Ctgf, C1s, Cxcl5, Reg3g and Ccl2 found in microarray (Paper II).

Efficacy, Specificity and Side-effects: Anti-angiogenic Effects of Selective Inhibition of NF- κ B Pathways (Paper III).

After a first confirmation that IMD0354 was effective in blocking NF- κ B in HUVEC culture and in rat cornea by suppressing its nuclear translocation, several *in vitro* and *in vivo* assays were used to determine the effect of IMD0354 on inflammation, wound healing and angiogenesis.

In the razor scratch wound assay²⁵⁹, IMD0354-treated HUVECs showed a dose-dependent reduction of migration rate.

The endocellular microfilament network, needed for cell migration, appeared to be disrupted by IMD treatment, as shown by staining for Actin-F (with phalloidin red). Scanning electron microscopy showed a reduction of cell filopodia.

The *in vitro* tube formation assay, where HUVECs are grown on Geltrex, showed partially disintegrated tubular structures, reduction of junction and tubule formation and decreased total tubule length with IMD0354 treatment, relative to controls.

Vessel sprouting evaluated *ex-vivo* using the aortic ring assay was inhibited by IMD0354 treatment.

Western blot and immunofluorescence demonstrated that IKK2 inhibition down-regulates VEGF-A and HIF-1 α expression in HUVECs.

In zebrafish embryo, IMD0354 showed a significant reduction in blood vessel development, process that is Vegf-dependent, showing that the suppression of VEGF-A by IKK2 studied *in vitro* is observable also *in vivo*. Immunofluorescence and western blot confirmed a decreased expression of Vegfa in this model.

In corneal neovascularization model, IKK2 inhibition by IMD0354 reduced inflammatory cell infiltration and limbal vasodilation at 5h, 48h and 96h, and decreased neovessel density *in vivo* at 96 h. However, it did not prevent neovascularization. The expression of several angiogenic mediators and NF- κ B downstream inflammatory chemokines (*Hif-1 α* , *Vegfa*, *Ccl2*, *Tnf*, *Cxcl5*, and *Cxcr2*) was attenuated by IKK2 inhibition, as showed by Western blot, IHC and qRT-PCR (Figure 15). Haematoxylin and eosin (H&E) staining indicated a milder cellular infiltration and neovessel formation, and reduced CD45 expression in the IMD0354-treated eyes.

NF- κ B pathway is crucial in many processes such as cell proliferation and survival; however toxicologic studies in vitro on HUVEC cultures and histologic analysis of rat liver in treated animals did not show signs of cell toxicity at the given doses.

Fig.15. *Quantitative Real Time PCR of inflammatory and neovascular factors in suture-stimulated rat corneas at 96 hours, showing decreased expression of Vegfa, Cxcl5, Ccl2 and Cxcr2 in corneas treated with NF- κ B inhibition by IMD0354, relative to sutured controls (CMC) and naïve corneas (Paper III).*

Wound Healing and the Endogenous Modulation of Inflammation and Angiogenesis in Cornea (Paper IV).

We studied the effects of blockade of Connexin 43 (Cx43), the gap junction α 1, through the mimetic peptide Gap27, on corneal wound healing and on inflammation in human corneal epithelial cell (HCEC) culture, in organ culture

(human cornea), and *in vivo* (rat cornea). The results show that blockade of Cx43 through Gap27 in cornea may accelerate epithelial wound healing in the early phase, but its prolonged use or use in deep stromal lesions could elicit undesirable inflammatory response.

More in detail, we observed that Gap27 accelerated cell migration *in vitro*: HCEC in the presence of Gap 27 migrated faster to close the gap than cells treated with the control substance (scGap27). Similarly, Gap27 accelerated wound closure *ex vivo* in cultured human corneas: wound closure started in Gap27 treated corneas at 6 h and was completed at 3 d but was delayed by days in scGap27.

Prolonged use of Gap27 enhanced the expression of inflammatory cytokines: IL-6 released from cultured corneas, assessed by ELISA, was reduced at 1 h compared to pretreatment levels in scGap27 but not in Gap27 treated corneas, where IL-6 levels were maintained; a sharp decline in VEGF-A release in scGap27 corneas was noted after 1 and 3 hours, but in Gap27 only after 3 hours.

In the *in vivo* corneal model, IVCN showed significantly greater density and migration distance of granulocytes into Gap27 treated corneas relative to scGap27 5 hours after suturing; at day 2 and 7, inflammation was detectable at and beyond the suture site. Gene expression of *Il-1 β* , *Tgf- β* , *Il-6* and *Tnf* did not differ between groups in the early phase of wound healing process (day 2), but at day 7, *Tgf- β* and *Tnf* were expressed at higher levels in Gap27 treated corneas. Gene expression for *Vegfa* did not differ between the groups at any time-point. No differences were observed in the average limbal vessel dilation *in vivo*; at 7 days, the vascularized area of the cornea did not differ between treatments.

4 DISCUSSION

This thesis focuses on inflammation-induced corneal neovascularization as a model for ocular angiogenesis, and studies its morphological, cellular, molecular and genetic events; moreover, it studies how these events are modulated by different pharmacological treatments and investigates the regulatory mechanisms and pathways active in ocular inflammatory angiogenesis. By doing so, the studies presented represent a contribution towards the quest for new, more effective treatments with fewer side-effects. The main hypothesis is that we need to address not only the angiogenic cascades but also the inflammatory component of the process.

Early Markers of Inflammatory Angiogenesis (Papers I, II, III)

In vivo longitudinal examination of corneal neovascularization confirmed that limbal vessel dilation, inflammatory cell infiltration of the stroma, and macrophage accumulation are early tissue markers of pre-angiogenic activity, clearly visible before the first angiogenic sprouts typically appear at the limbus in our model ²⁵⁸. Neutrophils and monocytes are the dominant subpopulations of infiltrating leukocytes during the early inflammatory response, producing a large number of inflammatory and pro-angiogenic chemokines, including VEGF ⁴³. The chemokines produced amplify the cascade inducing chemotaxis of leukocytes and polarize them towards a proangiogenic phenotype ²⁶⁰. Macrophages likely collect at the suture in an attempt to digest foreign material ²⁶¹, but even play a role in producing VEGF and other cytokines ¹⁶⁴. Limbal vessels dilate as part of the inflammatory response preceding neovascularization ²⁶².

Interestingly, limbal vessel dilation and macrophage infiltration in the pre-sprouting phase at day 2 appeared to correlate with final neovascular invasion at day 7, while the early migration of inflammatory myeloid cells and total myeloid cell presence did not appear to relate to the final development of neovessels, presumably because the release of their inflammatory and angiogenic mediators by degranulation is blocked by steroid treatment.

Modulation of Neovascular Response and its Early Markers by Anti-angiogenic and Anti-inflammatory Treatment (Papers I, II, III)

The early markers described could be addressed by initiating anti-angiogenic or anti-inflammatory treatment within two days after trigger, before new vessels are visible. At day 2, dexamethasone treatment strongly suppressed limbal vasodilation (Papers I, II); it suppressed as well chemotaxis of mature macrophages to the suture site despite an increased number of infiltrating myeloid cells (paper I); a similar effect was seen also in selective NF- κ B blockade (paper III). All three treatments showed as well a reduction of inflammatory cell infiltration of the aqueous humor relative to controls.

The dense infiltration of inflammatory myeloid cells in dexamethasone-treated corneas at all time-points was a surprising finding, even though already reported²⁶³, and might depend on active recruitment of specific anti-inflammatory leukocyte subsets¹⁹³, or prolonged neutrophil survival by glucocorticoids²⁶⁴.

In study I, dexamethasone suppressed corneal neovascularization by over 90%, versus 14% with anti-Vegf. Suppression of corneal neovascularization with dexamethasone has been reported in rodent models²⁶⁵⁻²⁶⁷. Anti-VEGF treatments, however, have had variable but comparatively poorer efficacy in both animal and clinical studies^{265,268-272}. In many studies bevacizumab or other molecules developed against human VEGF are used, while we have chosen a

species-specific antibody against rat Vegf; our qPCR analysis showed good penetration of the topical treatment; nevertheless, the treatment resulted in a very low efficacy. These results indicate limited effectiveness of the anti-VEGF treatment¹⁹⁷. Inhibition of early recruitment or maturation of macrophages by anti-angiogenic agents may also play an important role in later corneal neovascularization^{163,164,273,274}.

Characterization of VEGF-independent Pathways, and their Therapeutic Modulation (Papers I, II, III)

Since topical anti-angiogenic treatments were not effective in blocking cell recruitment, we investigated whether they might block their pro-angiogenic cytokine release^{165,274,275}. Gene expression modification of several major angiogenic, inflammatory and macrophage-related factors failed to explain the difference in anti-angiogenic effect observed between dexamethasone and anti-Vegf: both treatments influenced *Vegfa*, *Tnf*, *Fgf2* and the other cytokines analyzed in the same way (Paper I). An interesting observation was that the effect of anti-Vegf treatment was not restricted to *Vegfa*, but it downregulated several cytokines, for example *Il-6*, demonstrating the close association of IL-6 with VEGF^{270,276-278}. As a possible explanation for reduced efficacy of VEGF blockade, an up-regulation of Insulin-like Growth Factor 1 (IGF-1) and MIP-2 (CXCL2) after bevacizumab treatment has been reported in a corneal alkali burn model²⁷⁹, while tumor studies have demonstrated the potential of anti-VEGF treatment to induce alternative FGF-2-driven angiogenesis²⁸⁰.

In order to explain better the observed difference between dexamethasone and anti-Vegf, global gene expression analysis was performed in the same model at 48 h, with analysis of differentially-expressed genes (DEGs), biological processes and pathways (paper II). Dexamethasone consistently suppressed

more genes related to inflammation and angiogenesis processes and pathways than anti-Vegf treatment (511 more DEGs were detected in anti-Vegf than dexamethasone). Six genes were highly differentially expressed between these groups: *Cxcl5*, *Ccl2*, *Reg3g*, *C3*, *C1s* and *Ctgf*; in addition to these, strong suppression by dexamethasone was observed for *Cfi* and *Serpinb2* (Paper II). Interestingly, *Cxcl5*, *Reg3g*, *Ccl2* and *Serpinb2* were also among the most significantly suppressed genes during the endogenous restoration of corneal avascularity as reported in an earlier study by our group²⁸¹. Dexamethasone in this sense may mimic or activate natural mechanisms for maintaining corneal avascularity. *Cxcl5* and *Ccl2* are cytokines downstream of NF-κB, and were clearly suppressed by the selective inhibition of this pathway (Paper III).

REG3G (regenerating islet-derived protein 3 gamma) is a bactericidal peptide²⁸², reported to activate inflammation through NF-κB signaling pathways²⁸³; SERPINB2 (known as plasminogen activator inhibitor type 2 or PAI-2) is expressed by monocytes, macrophages and vascular endothelial cells and up-regulated during inflammation²⁸⁴. CXCL5 (also known as ENA-78, epithelial-derived neutrophil-activating peptide 78) and CCL2 (also known as MCP-1, monocyte chemoattractant protein-1) play an important role in the recruitment of neutrophils²⁸⁵,²⁸⁶,²⁸⁷ respectively monocytes²⁸⁸,²⁸⁹,²⁹⁰ and they both have direct effects on the migration and proliferation of vascular endothelial cells²⁹¹,²⁹²,²⁹³,²⁹⁴. CXCL5 and CCL2 are downstream of TNF and NF-κB, and several factors in this cascade are suppressed by dexamethasone²⁹⁵.

The suppression of chemotactic factors *Cxcl5* and *Ccl2* in our model is in apparent contradiction with the absence of inhibitory effect on the recruitment of inflammatory cells into the cornea, however, a possible explanation for this could be found in complement activation.

Microarray, immunofluorescence and Western blot indicated increased expression of complement factors *C3*, *C1s*, *C2* in dexamethasone treated corneas

compared to anti-Vegf. Increased gene expression of *C3* with glucocorticoids has been reported^{296 297}. In paper II, consistently with complement activation, a suppression of the inhibitory *Cfi* by dexamethasone was observed. The complement activation that results is able to recruit and activate inflammatory cells²⁹⁸. So, it could be hypothesized that dexamethasone suppressing CXCL5 and CCL2 achieves a potent anti-angiogenic effect, while instead activating the classical complement pathway in a chemokine-independent manner recruits inflammatory cells to mount an anti-microbial response. Notably, dexamethasone promotes antimicrobial activity *in vitro*²⁹⁹. A low-grade activation of complement cascade is believed to be normal in healthy cornea³⁰⁰, where it is functional for the protection against gram negative bacteria such as *Pseudomonas aeruginosa*³⁰¹. Complement system is clearly involved in pathologic choroidal angiogenesis in AMD³⁰² and laser-induced CNV³⁰³. Polymorphism of gene loci such as complement factor H and B greatly increases the risk of developing AMD^{304,305}. In cancer, complement may have a dual role in promoting tumor angiogenesis on the one hand, and exerts cytotoxic effect on tumor cells on the other³⁰⁶. In OIR, a model for ROP, *C3* and *C5* deficient mice had a higher rate of pathologic vessel growth³⁰⁷. In that study, it was suggested that activation of the complement system prevents retinal neovascularization, a process that appears to be mediated by polarization of macrophages towards an anti-angiogenic phenotype. The blockage of *Cfb* from the alternative pathway has been reported to enhance pathologic retinal neovascularization^{308,309}. These results, albeit in retinal models of hypoxia, are in line with our findings of higher expression of *C3* in corneas where the neovascular response is suppressed. The activation of complement in cornea seems thus beneficial, but its specific role needs to be confirmed (Figure 16).

The systematic investigation of genome-wide expression changes with different treatments of corneal inflammation and neovascularization elucidated several

candidate factors to target in order to complete an anti-VEGF blockade with the inhibition of VEGF-independent pathways, in order to render the therapy more effective.

Fig.16. Graphical summary (adapted from del Zoppo ³¹⁰) of the proposed mode of action of steroids in activating the classical complement pathway. The represented genes were differentially regulated by dexamethasone relative to the anti-Vegf treatment in the cornea model. Upregulated genes are shown in red color, while down regulated genes are shown in green color. The difference in fold change i.e. (fold change dexamethasone minus fold change anti-Vegf) is shown in parentheses, along with the corresponding p-value. The differentially expressed genes activate the classical complement pathway, leading to C3 upregulation, which in turn promotes inflammatory cell infiltration into the cornea. (Paper II).

Efficacy, Specificity and Side-effects: Anti-angiogenic Effects of Selective Inhibition of NF- κ B Pathways (Paper III).

Inflammation can trigger the development of corneal neovascularization^{258,311}, and its pathways are not adequately addressed by anti-VEGF treatment. On the other hand, a broad anti-inflammatory treatment like dexamethasone showed to perturb many biological processes and pathways (such as cell differentiation, regulation of apoptosis, cell adhesion, migration, leukocyte migration, the innate immune response, PI3K-Akt signaling, focal adhesion, and NF- κ B signaling) that might be crucial for the cell, creating a risk for serious side-effects.

In study III, we investigated targeting of the NF- κ B signaling pathway as a means to avoid broad inflammatory targeting and to specifically suppress the inflammatory cascade downstream of glucocorticoid receptor.

NF- κ B regulates the expression of many cytokines involved in the process³¹². Inhibition of the NF- κ B pathway resulted in reduced pre-angiogenic tissue activity, such as limbal vasodilation and inflammatory cell infiltration of the stroma. IMD0354 treatment suppressed *Ccl2* and *Cxcl5*, that were also the most suppressed cytokines by dexamethasone (Paper II) and by the endogenous mechanism of vessel regression²⁸¹; moreover IMD0354 suppressed *Cxcr2*, the receptor for *Cxcl5*, crucial in neutrophil recruitment³¹³. Besides anti-inflammatory effects, NF- κ B inhibition has also direct anti-angiogenic effects: the assays lacking active inflammation (HUVEC migration and tube formation, aortic ring sprouting, and zebrafish vascular development) indicated that IMD0354 has an inhibitory effect on VEGF production. A reduced expression of VEGF as a consequence of NF- κ B inhibition has previously been reported³¹⁴. The mechanisms of VEGF suppression might be mediated by HIF-1 α ^{315,316,317}, or by ABCA1, a regulator of cholesterol efflux from cells^{318,319,320}, both downregulated by IMD0354. A lower VEGF production interferes also with

actin F meshwork and cell motility ³¹⁴. The observed disruption of microfilaments might also be HIF-1 α -mediated independently of VEGF or hypoxia ³²¹, or ABCA1-mediated, since altered cholesterol would affect cytoskeleton composition ³²², or may even be a direct effect of IMD0354, since it has been shown to cause cell cycle arrest at the G₀-G₁ phase ³²³.

As NF- κ B plays a crucial role in many cellular processes, including cell survival and proliferation ³²⁴, its inhibition may lead to undesirable side effects, but the preliminary toxicologic data from our studies were encouraging.

Selective NF- κ B signaling pathway inhibition, in summary, resulted in a significant downregulation of multiple proinflammatory and proangiogenic factors in the cornea, as *Ccl2*, *Cxcl5*, *Vegfa*, *Tnf* and *Hif1 α* , and in reduction of pre-angiogenic tissue activity. Despite these effects, the angiogenic response was reduced, but not abolished. Targeting a single inflammatory non-VEGF pathway appears thus to be insufficient in order to suppress the neovascular response. A combined approach is more likely to be more effective.

Wound Healing and the Endogenous Modulation of Inflammation and Angiogenesis in Cornea (Paper IV).

Inflammation, angiogenesis and wound healing are strictly regulated processes. The inflammatory process brings to the site of injury a large amount of blood-derived immune cells and inflammatory factors needed to eliminate the threats. Among these cells, many macrophages, that can change polarization from pro-inflammatory to pro-angiogenic macrophages. The angiogenic process concludes the inflammatory phase and helps both in clearing up damaged tissue, as well as initiating the process of wound healing. These processes are, thus, closely interconnected and finely modulated in time and space in order to restore tissue homeostasis and avoid damage, involving endogenous inhibitory systems.

In Paper IV, endogenous inhibition of inflammation was studied during the physiologic wound healing response. The endogenous healing process is comparable to the stabilization phase obtained by removing the angiogenic stimulus, studied by our group in another doctoral thesis: the hypothesis being that an endogenous inflammatory as well as angiogenic switch could be activated and deactivated through specific pathways, and that coordinated delivery of anti-VEGF and anti-inflammatory therapy could transform active sprouts into mature, non-leaky and non-damaging functional capillaries ^{113,325}. Paper IV investigates if a therapy able to facilitate wound healing in cornea, could also act as an inhibitor of corneal inflammation and angiogenesis.

Corneal wound healing was studied using the connexin 43 (Cx43) mimetic peptide Gap27: this treatment accelerated the rate of wound closure of human corneal epithelium *in vitro* and *ex vivo*; however, no suppression on inflammatory or angiogenic mediators was observed. On the contrary, treatment of rat corneas with Gap27 after deep stromal injury resulted in an increased rate of early granulocyte infiltration, late gene expression of *Tnf* and *Tgf-β1*, and absence of inhibitory effect on pathological neovascularization.

Cx43 inhibition shows beneficial wound healing properties ^{178,180,187,189,190,326,327,328,329}, including beneficial effect on corneal epithelial wounds of diabetic rats *in vivo* ³³⁰ and chronic ulcers in diabetic patients ³³¹. Cx43 is expressed by macrophages ³³², neutrophils ³³³, lymphocytes ³³⁴ and mast cells ³³⁵. Immune response secondary to a tissue damage commences with an acute phase, when the neutrophils migrate to the wounded area and release inflammatory mediators affecting subsequent macrophage chemotaxis to the affected area. Cx43 mimetic peptides were reported to reduce inflammatory reactions to injury, however, the expression of *Tgf-β1* was not reduced after 1 day from treatment with siRNA against Cx43 in a rat model of endothelial injury ¹⁹⁰, and a significant increase of *Tnf* was reported in a diabetic rat model

of epithelial injury at day 3³³⁰. Similarly, a recent study confirmed that the expression of Tgf- β 1, along with collagen type I and III and Mmp-2, increases after 7 days of inducing a skin wound in Cx43 heterozygous knockout mice as compared to controls³³⁶. A possible explanation may be due to the ability of Gap27 to penetrate in the stroma and promote early inflammatory cell migration, as noted in the early hours after corneal stromal wounding in our model. However, other studies report opposite results, with reduced neutrophil and macrophage infiltration as a consequence of Cx43 inhibition, for instance with another peptide, ODN^{189 179 337}. Results, however, could be tissue-specific. Our results suggest that in corneal stromal wounds, inflammation can be promoted by Cx43 inhibition through cytokine expression, resulting in an associated accumulation of granulocytes and macrophages. The effects of Cx43 inhibition on cell migration and proliferation may therefore not be limited to epithelial cells around the wound site where cytokine expression may be beneficial in a transient phase, but it can also affect the inflammatory cells in the underlying stroma. A sustained stromal inflammatory response in the avascular cornea can upset the immune balance leading to pathological neovascularization. In line with our results in the cornea, using ODN to downregulate Cx43 gene expression, re-epithelialization was achieved in corneas with severe and persistent defects in five patients, however, vascularization of the corneas was evident after 1 year of treatment in two out of five patients¹⁸⁹. In human gingival fibroblasts treated with Gap27, an increased expression of VEGF was reported³³⁸.

Our results suggested no beneficial effects of Gap27 on suppression of VEGF or the subsequent vasodilation in limbal vessels, infiltration of granulocytes or vascularization after superficial or deep corneal injuries. Cx43 inhibition by Gap27 could be effective in accelerating the healing of epithelial wounds in an early phase, but prolonged use in stromal wounds could elicit an undesirable

inflammatory response, which could be deleterious especially for more invasive wounds, such as severe alkali burns, corneal transplantation and glaucoma infiltration procedures. A clear lesson is that it is not enough to close a wound to avoid inflammation and neovascularization. Despite these results, the idea of promoting endogenous inhibitory pathways of inflammation and angiogenesis warrants further investigation, since it might be a way to achieve more effective and specific therapies with fewer side-effects, i.e., more “physiologic” treatments.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis demonstrates that anti-inflammatory treatment with dexamethasone inhibits early angiogenic events such as early limbal capillary dilation and early macrophage recruitment in a corneal neovascularization model. These early inhibitory effects, not observed with anti-Vegf treatment, paralleled the inhibition of corneal neovascular sprouting. Both treatments, however, down-regulated several major angiogenic and inflammatory cytokines, without affecting neutrophil infiltration. Similar results were obtained with another anti-inflammatory treatment targeting specifically NF- κ B. To explain these findings, a systematic investigation of genome-wide expression changes with different treatments of corneal inflammation and neovascularization was undertaken, for the first time to our knowledge. Robust genome-wide analysis yielded candidate factors confirmed at the tissue and protein expression levels and provided gene lists as a resource for investigating novel targets for anti-inflammatory and anti-angiogenic therapy specific to the corneal milieu. The results show that the chemokines CXCL5, CCL2, REG3G and SERPINB2 are possible targets for inhibiting the early inflammatory response leading to corneal neovascularization, factors that anti-VEGF treatment does not appear to influence. Additionally, activation of the complement pathway, mostly the classical cascade, by dexamethasone was unexpected, and suggests a possible beneficial role that could be useful in therapy, and which warrants further investigation.

Dexamethasone inhibits many biological processes: its broad activity was shown to be more effective than anti-VEGF treatment in corneal neovascularization, but its utility is reduced by the many side-effects. A possible way to reduce undesired effects as suggested in the thesis, is addressing the cascade of interest downstream from glucocorticoid receptor, as was done in Paper III selectively blocking NF- κ B pathway; this has an effect also on downstream cytokines and

chemokines, like CCL2 and CXCL5, responsible for macrophage and neutrophil chemotaxis, the same that were strongly downregulated also by dexamethasone, as well as in the endogenous process of vessel regression²⁸¹. Moreover, NF- κ B inhibition downregulated VEGF, impairing blood vessel formation *in vitro* and *in vivo*, as well as several inflammatory cytokines, suppressed inflammatory cell infiltration, limbal vasodilation, and disrupted the microfilament meshwork and cell filopodia needed for endothelial cell migration. All these effects resulted in a decreased but not abolished corneal neovascularization. A single non-VEGF inflammatory pathway is thus not sufficient as a therapeutic target, but needs probably a combination therapy, for example together with anti-VEGF, or with multiple inflammation-related targets.

Another possibility to find more specific and effective therapies for neovascular diseases, but with fewer side-effects, is to achieve more “physiologic” treatments, promoting endogenous inhibitory pathways of inflammation and angiogenesis. Such pathways are activated for example by removal of the angiogenic stimulus¹¹³, but also by the wound healing process. An experimental agent facilitating corneal wound healing, confirmed its potential in accelerating corneal epithelial wound closure in an early phase, but showed in stromal wounds undesirable inflammatory response that may have derived from an equally potent acceleration of inflammatory cell migration. Clearly, it is not enough to close the wound in order to avoid inflammation and angiogenesis. These findings illustrate how inflammation, angiogenesis and wound healing are finely-tuned processes, interconnected with one another in a complex manner. Further approaches need to be investigated in order to mimic the natural process whereby pro-inflammatory and pro-angiogenic pathways are suppressed and anti-inflammatory pathways are activated.

In conclusion, in addition to targeting VEGF, selective targeting of inflammation-specific factors, possibly downstream of the main broad-acting

receptors, as well as complement modulation, may provide a route for improving the efficacy of anti-VEGF treatment while avoiding negative side effects of steroid use. Functional studies are needed in order to test the presented candidate targets, for example by blocking the function of the identified factors with blocking antibodies, siRNA, or in knock-out models. However, in this quest, efficacy and safety require us to warrant more attention to the possibility of promoting endogenous restoration of the physiological angiogenic balance.

Conclusions in bullet points

- Steroids are more effective than anti-VEGF in inflammatory corneal neovascularization due to multiple pathway inhibition.
- Many novel factors and pathways not addressed by anti-VEGF therapy were uncovered in corneal neovascularization and represent interesting targets for further study.
- A single inflammatory pathway is insufficient as anti-angiogenic therapy target
- Blockade of CCL2, REG3G or CXCL5 might have potential for adjuvant antiangiogenic therapy.
- Wound closure is not sufficient to avoid inflammatory angiogenesis in the cornea.
- Activation of complement cascade may have a beneficial role in modulating corneal neovascularization
- Functional studies are needed in order to test the presented candidate targets.

6 ACKNOWLEDGEMENTS

Neil Lagali, my main supervisor, someone with an even more international background than myself and with an impressive amount of miles gathered each year, a sharp mind and a kind person, but above all the catalyzer of everybody interested in research at the Department of Ophthalmology in Linköping.

Beatrice Peebo, my co-supervisor, who first invited me into our research group, always rich in new initiatives and new ideas, and whose sparkling energy is encouraging and inspiring.

Anthony Mukwaya, my fellow PhD student (now post-doc); thanks for fantastic collaboration during all the work done together, for invaluable help with microarray and with figures, for interesting exchange of views between a clinician and a molecular biologist.

Anton Lennikov, for fruitful collaboration and help, and for all the work done; for revolutioning everything at our lab, speeding up processes and publications, and for urging me to find time for research despite fullbooked clinics and despite my other duties (that could fill two working weeks every week...).

Maria Xeroudaki, for enthusiastic collaboration and hard work.

Lasse Jensen, for the advices and the comments on the study design and interpretation of results, for interesting discussions in our research meetings and journal clubs, and for teaching to critically review published scientific literature.

Per Fagerholm, for establishing our research group at the Department of Ophthalmology of Linköping's University.

Christina Frennesson, for reminding everybody how important research is, and how our daily practice as physicians should always follow evidence from research.

Sven Jarkman, my appreciated chief for several years, the one who convinced me to move to Linköping, and who always had time to talk despite gathering too much work on himself (whom have I learned from?).

Zaheer Ali, for assistance and contribution with zebrafish studies.

Hossein Elbadawy, for the interesting collaboration with Banca degli Occhi di Venezia.

Maria Koulikovska, for help in my first steps in the laboratory.

Catharina Traneus Röckert and **Camilla Hildesjö**, for help with histologic sections and stainings.

Monica Johansson, Annette Delby, Karin Hillmo Klevebrandt, Christina Gylling, and all the staff at the **Department of Ophthalmology at the University of Linköping**, for supporting my research and my clinical work in many ways, as well as being there also outside work.

My family: my parents, for raising me and giving me the possibility to study and to reach this goal, in particular my father Ottavio for teaching me a deep ethics of work and sense of duty, and my mother Leila for all the love and support, and for transmitting me faith; my sister Cristina and my brother Paolo for being so close to me despite 3000 km distance, my nephew Federico, curious and already deeply interested in science. I here also remember my grandmother, that calling me “dottore” when I was a little child might have influenced my choices in life, and my grandfather, that showed what caring of beloved ones means in practice, without words needed.

Lastly but not least, all my praise goes to the Creator of the universe and of life, the amazing object of our research; the more we investigate, the more we understand how fascinating complicated and fine-tuned reality is. And, though, I am cared of.

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FACULTY OF MEDICINE AND HEALTH SCIENCES

Linköping University Medical Dissertation No. 1685, 2019
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