# Retinal oximetry and age-related macular degeneration

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UNIVERSITY OF ICELAND
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# Súrefnisbúskapur sjónhimnu og aldursbundin augnbotnahrörnun

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# Ágrip


**Tilgangur**

Vot aldursbundin augnbotnahrörnun er algengasta orsök sjónskerðingar og blindu í vestrænum ríkjum en sjóntapið tengist sjúklegri nýæðamyndun og blæðingum í augnbotninum sem að lokum leiða til örvefsmyndunar. Nýæðamyndun er almennt talin tengjast blóðþurrð og súrefnisskorti sem hvetur til myndunar vaxtarþátta. Einn þeirra er vaxtarþáttur æðaþels (e. vascular endothelial growth factor, VEGF) en meðferð við votri augnbotnahrörnun byggist að stærstum hluta á mótefnum gegn VEGF sem sprautað er inn í glerhlaup augans.

Út frá þessum athugunum og niðurstöðum annarra rannsókna hafa grunsemdir vaknað um að í votri augnbotnahrörnun sé um að ræða truflun á súrefnisbúskap sjónhimnunnar. Takmörkuð tækni hefur verið í boði til að mæla súrefnismettun í augnbotnum manna, þar til rannsóknarhópur okkar þróaði slíkan tækjabúnað nýlega.

Tilgangur rannsóknanna í þessari ritgerð er að kanna hvort súrefnismettun augnbotna sé óeðlileg í votri augnbotnahrörnun. Til að ná því takmarki var í fyrsta lagi mæld súrefnismettun í sjónhimnuæðum heilbrigðra til að staðla tækni við súrefnismælingar og fá heilbrigðan samanburðarhóp. Í öðru lagi var mæld súrefnismettun í sjónhimnuæðum sjúklinga með vota augnbotnahrörnun. Að lokum var kannað umfang votrar augnbotnahrörnunar og mótefnameðferðarinnar til að meta áhrif sjúkdómsins á heilbrigðisþjónustu á Íslandi.

**Aðferðir**

Súrefnismælingar sjónhimnu heilbrigðra og sjúklinga með vota augnbotnahrörnun:

Þátttakendur í rannsóknum á súrefnismettun í sjónhimnu voru alls 120 heilbrigðir á aldrinum 18-80 ára og 46 sjúklingar á aldrinum 66-95 ára með vota augnbotnahrörnun sem ekki höfðu áður fengið sprautumeðferð í glerhlaup augans.

Súrefnismælirinn er byggður á augnbotnamyndavél. Með áföstum myndeili er samtímis unnt að taka tvær augnbotnamyndir á tveimur mismunandi bylgjulengdum, 570 og 600 nm, inn á tvær stafrænar myndavélar. Sérhæfður hugbúnaður reiknar súrefnismettun sjónhimnuæða út frá mismunandi ljósendurkasti bæði slag- og bláæðlinga á þessum tveimur bylgjulengdum.

Faraldsfræði votrar augnbotnahrörnunar á Íslandi:

Þátttakendur í framsýnu faraldsfræðirannsókninni voru allir 60 ára og eldri með vota augnbotnahrörnun á leið í sína fyrstu mótefnameðferð með

ranibizumab sem sprautað var í glerhlaup augans. Alls voru 439 einstaklingar sem hófu meðferð á tímabilinu mars 2007 til desember 2009. Árlegt nýgengi votrar augnbotnahrörnunar á Íslandi var áætlað og fjöldi sprautumeðferða á fyrstu 12 mánuðum eftir upphaf meðferðar var metinn fyrir sérhvert auga í meðferðinni og fyrir þýðið í heild.

**Niðurstöður**

Súrefnismælingar sjónhimnu heilbrigðra og sjúklinga með vota augnbotnahrörnun:

Súrefnismettun sjónhimnuæða í heilbrigðum var 92,2±3,7% (meðaltal± staðalfrávik) í slagæðlingum og 55,6±6,3% í bláæðlingum. Mismunur súrefnismettunar slag- og bláæðlinga var 36,7±5,4%. Súrefnismælirinn reyndist áreiðanlegur og sýndi engan mun á súrefnismettun í hægra og vinstra auga í slagæðlingum (p=0,30) og bláæðlingum (p=0,07). Staðalfrávik endurtekinna mælinga í sömu æð var 1,0% í slagæðlingum og 1,4% í bláæðlingum. Súrefnismettun í neðri gagnauga (e. temporal) fjórðungi sjónhimnunnar mældist marktækt lægri í bæði slagæðlingum (p<0,0001) og bláæðlingum (p<0,0001) en í öðrum fjórðungum. Súrefnismettun í slag-æðlingum var stöðug með aldri í körlum (p=0,30) og í konum (p=0,23). Fyrir sérhver 10 ár hækkandi aldurs lækkaði súrefnismettun í bláæðlingum um 1,9±0,6% (meðaltal±staðalskekkja meðaltalsins) hjá körlum (p=0,003) og 0,7±0,4% hjá konum (p=0,068). Mismunur súrefnismettunar í slag- og bláæðlingum jókst hins vegar fyrir hver 10 ár um 1,5±0,5% hjá körlum (p=0,004) og 1,0±0,4% hjá konum (p=0,007). Fyrir hverja 10 mmHg hækkun gegnumflæðisþrýstings (e. perfusion pressure) auga hækkaði súrefnismettun um 0,9±0,4% í slagæðlingum (p=0,024) og um 1,2±0,7% í bláæðlingum (p=0,075).

Í samanburði við heilbrigða viðmiðunarhópinn var súrefnismettun í sjúklingum með vota augnbotnahrörnun 91,5±4,5% (meðaltal±staðalfrávik) í slagæðlingum, 54,4±9,0% í bláæðlingum og mismunur súrefnismettunar slag- og bláæðlinga var 37,1±7,1%. Línuleg aðhvarfsgreining á gögnunum sýndi jafnframt að með auknum aldri hækkaði súrefnismettun bláæðlinga hjá sjúklingum með vota augnbotnahrörnun en lækkaði hjá heilbrigðum og var þessi munur milli hópanna marktækur (p=0,0003). Enn fremur mátti sjá marktækan mun milli hópanna fyrir mismun súrefnismettunar slag- og bláæðlinga (p=0,0017) en með auknum aldri lækkaði mismunur súrefnismettunar slag- og bláæðlinga hjá sjúklingunum á meðan þessi mismunur jókst hjá heilbrigðum. Enginn marktækur munur var á sambandi súrefnismettunar slagæðlinga og aldurs milli hópanna tveggja (p=0,075).

Faraldsfræði votrar augnbotnahrörnunar á Íslandi:

Árlegt nýgengi votrar augnbotnahrörnunar hjá Íslendingum 60 ára og eldri var 0,29%. Með auknum aldri jókst nýgengið frá 0,06% í sjúklingum yngri en 70 ára upp í 0,96% hjá þeim sem voru 85 ára og eldri.

Á fyrstu 12 mánuðum mótefnameðferðar með ranibizumab fékk sérhvert sjúkt auga á bilinu 3-12 sprautur í glerhlaup augans, eða að meðaltali 5,0±2,3 meðferðir. Miðað við þennan fjölda sprauta í hvert auga með nýgreinda vota augnbotnahrörnun er unnt að áætla að fyrir hverja 100 000 einstaklinga 60 ára og eldri á Íslandi og í sambærilegu þýði væri þörf á um það bil 2 400 glerhlaupsmeðferðum ár hvert.

**Ályktanir**

Tiltölulega lítill breytileiki er í súrefnismettun í sjónhimnuæðum manna og breytingar með aldri eru smávægilegar, þótt marktækar séu. Tæknin virðist áreiðanleg og bæði tæknilegur og líffræðilegur breytileiki er lítill. Marktækur munur er á súrefnismettun sjónhimnuæða í heilbrigðum og augum sjúklinga með vota augnbotnahrörnun. Þó verður ekki fullyrt að um súrefnisskort sé að ræða í þessum augum; frekari rannsóknir þarf til að skera úr um það.

Vot augnbotnahrörnun er algeng á Íslandi og umfang mótefnameðferðar við sjúkdómnum mikið og vaxandi og á það væntanlega einnig við um nágrannalönd.

**Lykilorð**

Sjónhimna, súrefnismælingar, aldursbundin augnbotnahrörnun, mótefni gegn vaxtarþætti æðaþels, nýgengi.

# Abstract

## Purpose

Neovascular age-related macular degeneration (AMD) is the leading cause of visual impairment and blindness in elderly persons in the industrialised areas. This impairment in vision is caused by pathological neovascularisation and hemorrhages in the fundus with subsequent fibrovascular scar formation. Neovascularisation is generally related to ischaemia and hypoxia, which induces formation of growth factors. One of which is vascular endothelial growth factor (VEGF) but the main treatment of neovascular AMD is with antibodies against VEGF.

This, as well as results from other studies, suggests that the retinal oxygen metabolism is disturbed in neovascular AMD. Limited technology has been available to measure oxygenation of the fundus in humans, until recently when our research group developed such apparatus.

The aim of the studies in this thesis is to determine if retinal vessel oxygen saturation is abnormal in patients with neovascular AMD. To reach that goal, the retinal oxygen saturation was first measured in healthy persons to standardise the retinal oximetry imaging technique and establish a normal control group. Subsequently, the retinal oxygen saturation was measured in patients with neovascular AMD. Finally, the scope of neovascular AMD and its intravitreal anti-VEGF treatment in Iceland was assessed to evaluate the health-care impact of the disease in Iceland.

## Methods

Retinal oximetry in health and neovascular AMD:

The retinal oximetry studies included 120 healthy subjects aged 18-80 years and 46 treatment-naïve neovascular AMD patients aged 66-95 years. The retinal oximeter is based on a fundus camera. With a coupled image splitter it is possible to simultaneously capture two retinal images at two different wavelengths, 570 and 600 nm, with two separate digital cameras. Specialised software calculates the retinal vessel oxygen saturation from the different reflection of light in both arterioles and venules at these two different wavelengths.

Epidemiology of neovascular AMD in Iceland:

The population-based AMD incidence study was a prospective study on 439 consecutive patients aged 60 years and older with neovascular AMD that started intravitreal anti-VEGF treatment with ranibizumab for neovascular AMD in Iceland from March 2007 to December 2009. The estimated annual

incidence of neovascular AMD in Iceland was determined as well as the number of intravitreal injections required for each treated eye and on a population scale.

**Results**

Retinal oximetry in health and neovascular AMD:

Retinal vessel oxygen saturation in the healthy subjects was 92.2±3.7% (mean±standard deviation (SD)) in arterioles and 55.6±6.3% in venules. The arteriovenous difference of oxygen saturation was 36.7±5.4%. The oximeter was reliable with no significant difference in oxygen saturation between right and left eyes in arterioles (p=0.30) and venules (p=0.07). The standard deviation of repeated oximetry measurements in a single (same) vessel was 1.0% in arterioles and 1.4% in venules. The inferotemporal quadrant had the lowest oxygen saturation in arterioles (p<0.0001) and venules (p<0.0001). The oxygen saturation of arterioles was stable with age in males (p=0.30) and females (p=0.23). The oxygen saturation of venules decreased by 1.9±0.6% (mean±standard error of mean (SEM)) per 10 years of age in males (p=0.003) and by 0.7±0.4% in females (p=0.068). The arteriovenous difference increased by 1.5±0.5% per 10 years in males (p=0.004) and 1.0±0.4% (p=0.007) in females. For every 10 mmHg increase in ocular perfusion pressure, oxygen saturation increased by 0.9±0.4% in arterioles (p=0.024) and by 1.2±0.7% in venules (p=0.075).

In comparison to the healthy group, the retinal oxygen saturation of the AMD patients was 91.5±4.5% (mean±SD) in arterioles, 54.4±9.0% in venules and the arteriovenous difference was 37.1±7.1%. The regression analyses showed a highly significant difference in the venules (p=0.0003), in which the venular oxygen saturation increased with age in AMD patients but decreased with age in the healthy subjects. Similary, a significant difference was found for the arteriovenous difference between the AMD patients and the healthy (p=0.0017), where the arteriovenous difference decreased with age in AMD patients but increased with age in healthy. No significant difference was found between the groups in the retinal oxygen saturation of arterioles (p=0.075).

Epidemiology of neovascular AMD in Iceland:

The annual incidence of neovascular AMD in the Icelandic population of 60 years and older was 0.29%. With advancing age, the incidence increased from 0.06% in patients less than 70 years old to 0.96% for those aged 85 years and older.

During the first 12 months after initiation of intravitreal ranibizumab treatment, each eye received 5.0±2.3 (mean±SD; median 4; range 3-12) injections. With this rate of injections, it can be expected that around 2 400 intravitreal ranibizumab injections would be required each year for treatment

of neovascular AMD per 100 000 persons aged 60 years and older in Iceland and comparable Caucasian populations.

**Conclusions**

Variability in the retinal vessel oxygen saturation is relatively small in human eyes and differences with age are minor, though significant. The oximetry technique appears reliable and both technical and biological variation is small. The results suggest that significant difference exists in retinal vessel oxygen saturation between healthy persons and patients with neovascular AMD. However, it cannot be confirmed if retinal hypoxia is present in patients with neovascular AMD; further studies are required to determine this.

Neovascular AMD is common in Iceland and the burden of its effective treatment is large and growing in Iceland as well as in comparable populations.

**Keywords**

Retina, oximetry, age-related macular degeneration, antibodies for vascular endothelial growth factor, incidence.

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**Conflicts of interest:**

None.

# Contents

# List of abbreviations

| | |
|---|---|
| A | Age |
| AMD | Age-related macular degeneration |
| ANCHOR | Anti-VEGF antibody for the treatment of predominantly classic choroidal neovascularisation in age-related macular degeneration trial |
| ANCOVA | Analysis of covariance |
| ANOVA | Analysis of variance |
| AREDS | Age-Related Eye Disease Study |
| AV | Arteriovenous |
| BCVA | Best corrected visual acuity |
| CATT | Comparison of AMD treatments trials |
| CI | Confidence interval |
| CNV | Choroidal neovascularisation |
| dL | Deciliter |
| DP | Diastolic blood pressure |
| G | Gender |
| HARBOR | Phase III, double-masked, multicenter, randomized, active treatment-controlled study of the efficacy and safety of 0.5 mg and 2.0 mg ranibizumab administered monthly or on an as-needed basis (PRN) in patients with subfoveal neovascular age-related macular degeneration |
| Hb | Deoxygenated haemoglobin |
| $HbO_2$ | Oxygenated haemoglobin |
| HIF | Hypoxia-inducible factor |
| HTN | Hypertension |
| I | Light intensity on a vessel |
| $I_0$ | Light intensity of background, to the side of vessels |
| IOP | Intraocular pressure |
| IVAN | Inhibition of VEGF in age-related choroidal neovascularisation trial |
| MAP | Mean arterial pressure |

| | |
|---|---|
| MARINA | Minimally classic/occult trial of the anti-VEGF antibody ranibizumab in the treatment of neovascular age-related macular degeneration |
| mmHg | Millimetres of mercury (unit of pressure) |
| mL | Milliliter |
| n | Number (of subjects studied) |
| OCT | Optical coherence tomography |
| OD | Optical density (measure of light absorbance) |
| ODR | Optical density ratio (ratio of two optical densities) |
| OPP | Ocular perfusion pressure in mmHg |
| p | Probability value |
| $PaO_2$ | Partial pressure of dissolved oxygen in blood |
| PRN | Pro re nata |
| PrONTO | Prospective optical coherence tomography (OCT) imaging of patients with neovascular age-related macular degeneration (AMD) treated with intraocular ranibizumab |
| SD | Standard deviation |
| SEM | Standard error of mean |
| SG | Study group |
| SP | Systolic blood pressure |
| $SaO_2$ | Oxygen saturation in % |
| $SpO_2$ | Oxygen saturation in % from finger pulse oximeter |
| SS | Smoking status |
| VEGF | Vascular endothelial growth factor |

# List of figures

# List of tables

# List of papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I. Geirsdottir, A., Palsson, O., Hardarson, S. H., Olafsdottir, O. B., Kristjansdottir, J. V., Stefánsson, E. (2012). Retinal vessel oxygen saturation in healthy individuals. *Invest Ophthalmol Vis Sci* 53(9): 5433-5442.

II. Palsson, O., Geirsdottir, A., Hardarson, S. H., Olafsdottir, O. B., Kristjansdottir, J. V., Stefánsson, E. (2012). Retinal oximetry images must be standardized: a methodological analysis. *Invest Ophthalmol Vis Sci* 53(4): 1729-1733.

III. Geirsdottir, A., Hardarson, S. H., Olafsdottir, O. B., Stefánsson, E. (2014). Retinal oxygen metabolism in exudative age-related macular degeneration. *Acta Ophthalmol* 92(1): 27-33.

IV. Geirsdottir, A., Jonsson, O., Thorisdottir, S., Helgadottir, G., Jonasson, F., Stefánsson, E., Sigurdsson, H. (2012). Population-based incidence of exudative age-related macular degeneration and ranibizumab treatment load. *Br J Opthalmol* 96(3): 444-447.

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# Declaration of contribution

**Paper I: Retinal vessel oxygen saturation in healthy individuals.**

I took part in planning this study. I examined (almost all) the participants. I analysed all oximetry images and contributed to the statistical analyses. I wrote the manuscript draft and participated in all subsequent revisions.

**Paper II: Retinal oximetry images must be standardized: a methodological analysis.**

I took part in planning this study. I examined (almost all) the participants. I took part in interpretation of the results and revision of the manuscript.

**Paper III: Retinal oxygen metabolism in exudative age-related macular degeneration.**

I took part in planning this study. I analysed all oximetry images and contributed to the statistical analyses. I participated in interpretation of the results. I wrote the manuscript draft and participated in all subsequent revisions.

**Paper IV: Population-based incidence of exudative age-related macular degeneration and ranibizumab treatment load.**

I took part in planning this study. I analysed all data and contributed in interpretation of the results. I wrote the manuscript draft and participated in its revision.

# 1 Introduction

## 1.1 Retina

The unique construction of the eye with the generally transparent cornea and lens allows direct visualisation of the retina, which lines the back of the eye. The retina is part of the central nervous system and consists of several cell layers (**Figure 1**) that form an interdependent anatomical, functional and metabolic network in the eye. The retinal layers are also transparent, allowing light to pass through the retina to the photoreceptors (rods and cones), which comprise the outermost layer of retina, and are the neurons that are directly sensitive to light (Guyton and Hall, 2000).

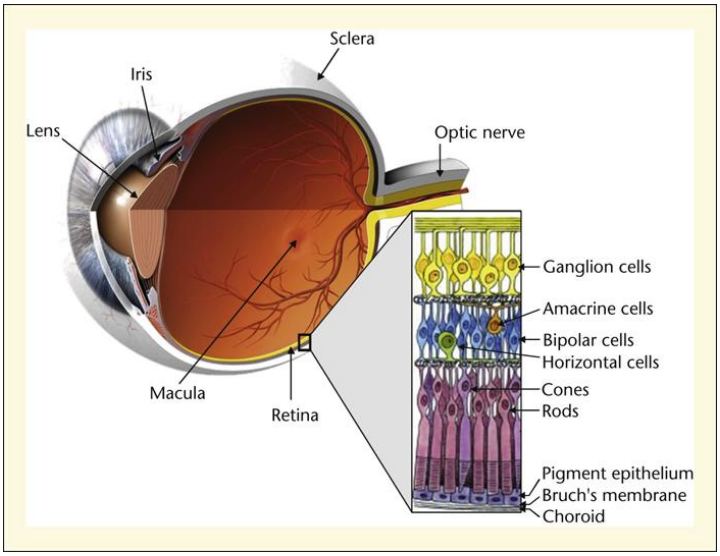


**Figure 1**. A cross-section of the eye with a schematic drawing of the layers of retina and the retinal pigment epithelium, the Bruch's membrane and the choroid. Reprinted from OCL, 18(5), Bretillon et al., Needs in omega 3 and ocular pathologies, 279-283, ©2011, with permission of Lionel Bretillon.

The macula lutea, or simply macula, measures about 5.5 mm in diameter and is centered between the optic disc nasally and the temporal vascular arcades superiorly and inferiorly (Schubert, 2012), as seen in **Figure 2**. In the very center of the macula is the fovea, a small depression in the inner retina composed of closely packed photoreceptors, majority of which are cones. In contrast to the rods, which function well in dim lighting and provide the black-and-white vision, the cones require well-lit environment to function and the cones support the perception of colour. Therefore, with the fovea packed with

cones, the fovea is the most sensitive part of the retina and responsible for the sharp visual acuity (Guyton and Hall, 2000).

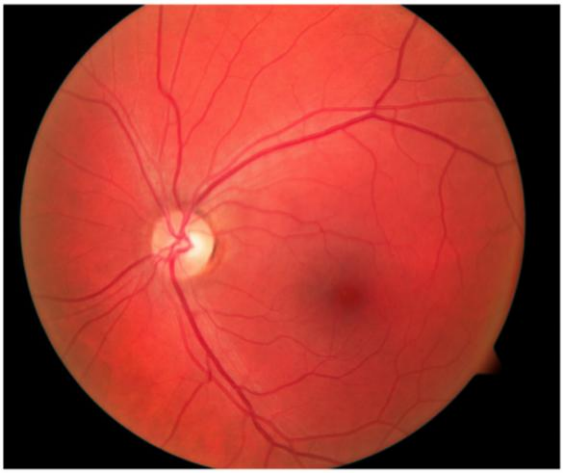

**Figure 2.** The normal fundus of a left eye showing the optic disc with the emerging retinal vessels and the macula with the fovea in the center.

## 1.2 Retinal oxygenation

Oxygenation of the retina is from two vascular systems, the retinal circulation and the choroidal circulation. Both vascular systems of the eye originate from the ophthalmic artery, a branch from the internal carotid artery. The ophthalmic artery gives rise to the central retinal artery and the ciliary arteries. The central retinal artery is the source of the retinal circulation. It enters the eye through the optic nerve and supplies oxygen to the inner retina by dividing into four (usually) major branches of arterioles at the optic disc: the superotemporal, the inferotemporal, the superonasal and the inferonasal vascular arcades (**Figure 2**). The four vascular arcades each supply blood to one quadrant of the retina. The retinal arterioles branch repeatedly to form the retinal capillaries. The retinal capillaries form an interconnecting network in the retina (**Figure 3**), which is normally organised in two layers. The inner layer is found in the nerve fiber layer and the ganglion cell layer but the outer layer is located in the inner nuclear layer and the plexiform layers (Hayreh, 2011; Kong et al., 2010; Pournaras et al., 2008).

Additional superficial layer of capillaries can be found in the peripapillary area and running alongside the first part of the temporal arterioles, ultimately anastomosing with each other and the underlying capillaries (Pournaras et al., 2008). In contrast, no capillaries are found in the nearest vicinity of the retinal arterioles and in the fovea (the foveal avascular zone) and only one layer of capillaries is found closely surrounding the fovea. The same is true in the periphery of the retina, where only one layer of capillaries is found and then an

avascular zone at the extreme periphery (Hayreh, 2011; Pournaras et al., 2008). The outer layers of the retina, including the highly oxygen and energy demanding photoreceptors, are also avascular, and as other avascular zones of the retina, are dependent on the underlying chorioidal circulation. The retinal capillaries ultimately merge and form the retinal venules that generally run alongside the retinal arterioles, and finally combine into the central retinal vein, which leaves the eye adjacent to the central retinal artery within the optic nerve.

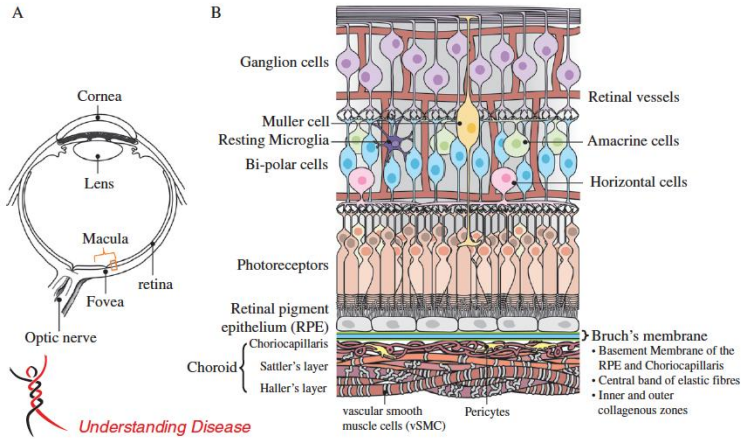


**Figure 3.** Schematic diagrams of (A) the eye and (B) vascular systems of retina and choroid. The retinal vascular system consists of retinal arterioles and venules connected by a network of capillaries organized in layers. The choroidal vascular system consists of three layers; the large-caliber arteries of the outer Haller layer, the medium-sized arteries and arterioles of the Sattler layer and the innermost layer of the choriocapillaris. Reprinted from J Pathol, 232(2), van Lookeren Campagne et al., Mechanisms of age-related macular degeneration and therapeutic opportunities, 151-164, ©2014, with permission of Genentech and John Wiley & Sons Ltd.

Blood enters the choroidal circulation through the posterior ciliary arteries. The choroidal vascular system consists of three layers (see **Figure 3**); the large-caliber arteries of the outer Haller layer, the medium-sized arteries and arterioles of the Sattler layer and the innermost layer of the choriocapillaris (Nickla and Wallman, 2010). The choriocapillaris is a network of relatively wide capillaries separated from the retina by the Bruch's membrane in a healthy retina. Oxygen diffuses from choriocapillaris through Bruch's membrane and retinal pigment epithelium to reach the outer retina. The choroidal circulation is vital for the oxygen supply of the photoreceptors in the outer retina. After the blood passes through the choriocapillaris, it is collected in venules, which coalesce into collecting channels of the vortex veins. Of note, in up to half of the population (bilaterally in about 15%), one or more cilioretinal arteries can supply part of the retina (Justice and Lehmann, 1976), most often a portion of the macular area, although cases where cilioretinal

arteries have supplied the entire retina have been seen (Hegde et al., 2006).

Retinal and choroidal circulations are anatomically and functionally greatly different. The retinal circulation is characterised by very low blood flow (Alm and Bill, 1973) and particularly high oxygen extraction with arteriovenous difference of oxygen saturation above 30% (Schweitzer et al., 1999). On the contrary, the choroidal circulation has very high blood flow, providing over two-thirds of the total ocular blood flow (Alm and Bill, 1972), but it has extremely low oxygen extraction, or as low as 3% (measured in cats) (Alm and Bill, 1970). The choroid also has the highest oxygen levels in the eye, but the level of oxygen falls dramatically across the outermost retina, thereby creating a large concentration gradient of oxygen towards the retina and inner segments of the photoreceptors (Linsenmeier, 1986). These extremes in oxygen tension expose the retinal pigment epithelium cells to a highly oxidized environment and increase their risk of oxidative damage.

The boundary between the oxygen diffusion from the choriocapillaris and the retinal vascular supply varies in relation to the topographic location within the retina, the thickness of the retina and the metabolic state, which is affected by illumination. The fovea is located in the small foveal avascular zone and therefore, receives most of its blood supply from the underlying choroidal circulation. The amount of light present also affects the oxygen diffusion in the eye as the retina uses more oxygen in dark than in light (Hardarson et al., 2009a; Stefánsson et al., 1983).

Generally, regulation of retinal blood flow has been considered more effective than that of the choroidal blood flow. Although the retinal vessels have no autonomic innervation like the choroidal vessels, the retinal circulation has a highly efficient autoregulation, thereby maintaining blood flow despite alterations in ocular perfusion pressure. Ocular perfusion pressure (*OPP*) can be calculated according to Equation 1:

$$OPP = MOAP - IOP = \frac{2}{3} MAP - IOP, \qquad Equation\ 1$$

where MOAP is the mean ophthalmic arterial pressure, MAP is the mean brachial arterial pressure and IOP is the intraocular pressure. Mean ophthalmic arterial pressure is arbitrarily defined as two-thirds of the mean brachial arterial pressure (Medeiros et al., 2007). Mean arterial pressure is defined as the average arterial pressure during a single cardiac cycle and calculated from cardiac output, systemic vascular resistance and central venous pressure. However, at normal resting heart rates, mean arterial pressure can be closely approximated according to Equation 2 using the more accessible systolic pressures (*SP*) and diastolic pressures (*DP*). Pulse pressure (*PP*) is the difference between the systolic and diastolic pressures.

$$MAP \cong DP + \frac{1}{3}PP \cong DP + \frac{1}{3}(SP - DP) \cong \frac{2}{3}DP + \frac{1}{3}SP. \qquad \textit{Equation 2}$$

The exact nature of the retinal autoregulation is still not fully explained but interaction of metabolic and myogenic mechanisms are known to play a role (Pournaras et al., 2008). Changes in oxygen tension, metabolic needs of the tissue and ocular perfusion pressure trigger alteration of the vascular resistance with dilation and constriction of the retinal arterioles. However, studies have also shown that the pericytes in the retinal capillaries play a role in this autoregulation of the microvascular circulation due to their contractile capacity (Anderson and Davis, 1996; Anderson, 1996). Inhalation of increased concentrations of oxygen or carbon dioxide has been shown to affect retinal blood flow. Hyperoxia leads to vasoconstriction (Palkovits et al., 2014; Riva et al., 1983) and decreased blood flow in retinal arterioles (Stefánsson et al., 1988a) and retinal venules (Palkovits et al., 2014), whereas hypercapnia causes vasodilation (Venkataraman et al., 2008). The retinal circulation must also maintain adequate blood flow despite changes in ocular perfusion pressure, which is, as described earlier, affected by the systemic blood pressure and the intraocular pressure. Decrease in blood pressure or an increase in the intraocular pressure leads to lower ocular perfusion pressure, which in turn leads to dilation of retinal arterioles. Similarly, higher ocular perfusion pressure leads to constriction of retinal arterioles (Pournaras et al., 2008).

Traditionally, choroidal circulation was not believed to have any autoregulation but rather being regulated through the autonomic innervation, where various animal models showed a linear relationship between the choroidal blood flow and ocular perfusion pressure (Alm and Bill, 1973; Yu et al., 1988). Later, choroidal blood flow has been shown to have some autoregulatory capacity in response to changes in mean arterial pressure, possibly through the sympathetic (vasoconstricting) and parasympathetic (vasodilating) innervation of the choroidal vessels (Nickla and Wallman, 2010) but this capacity appears to be less efficient with changes in intraocular pressure (Kiel and Shepherd, 1992; Polska et al., 2007; Schmidl et al., 2011a). Concentration of carbon dioxide and carbon monoxide can affect choroidal blood flow to some extent but the concentration of oxygen seems to have little effect (for review see Schmidl et al., 2011b).

## 1.3 Retinal oximetry

Measuring oxygen saturation of retinal vessels can give valuable information on the oxygen metabolism of the retina, which is a highly metabolically active tissue as discussed in chapter 1.1. Measurements of retinal oxygenation may also increase the understanding of retinal oxygen metabolism in health and disease as discussed further in chapters 1.3.4 and 1.3.5. Knowledge of the

retinal oxygenation in diseases can possibly give better insight into their pathophysiology as well as supporting their diagnosis and management.

### 1.3.1 Physiology of oxygenation

Oxygen is carried to the retina in the arteries and arterioles. The majority (98%) of the oxygen is bound to haemoglobin as oxyhaemoglobin, or saturated haemoglobin, but a fraction (2%) is dissolved in the plasma, accounting for the partial pressure of oxygen (Guyton and Hall, 2000). A dynamic equilibrium exists between the oxyhaemoglobin and the nonbonded haemoglobin, represented by the oxygen-haemoglobin dissociation curve, see **Figure 4**.

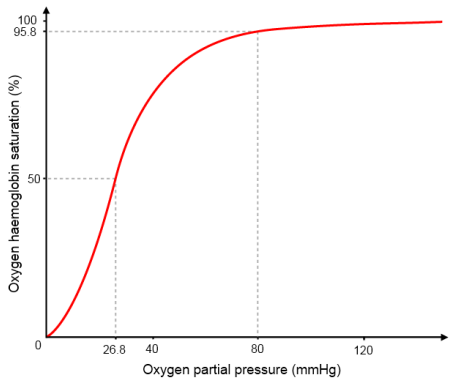


**Figure 4.** Oxygen-haemoglobin dissociation curve. The curve describes the relationship between the available oxygen (oxygen partial pressure) and the amount of oxygen carried by haemoglobin (oxygen haemoglobin saturation). Public domain figure. https://commons.wikimedia.org/wiki/File:Hb_saturation_curve.png (labels clarified).

The oxygen-haemoglobin dissociation curve has a characteristic sigmoid shape due to the co-operative binding of oxygen to haemoglobin, in which the affinity for oxygen increases with successive binding of oxygen molecules. According to the oxygen dissociation curve, the higher the partial pressure of dissolved oxygen in blood, the more oxygen molecules will bind to available haemoglobin, resulting in higher haemoglobin oxygen saturation. Thus, the haemoglobin oxygen saturation depends on the concentration of dissolved oxygen molecules but also on the amount of haemoglobin present in blood as explained below. Moreover, several factors can affect this relationship and shift the curve to left or right, like changes in pH, temperature and carbon dioxide concentration (Guyton and Hall, 2000). The haemoglobin oxygen saturation ($SaO_2$) is generally defined as the percentage of oxyhaemoglobin in blood according to Equation 3:

$$SaO_2 = \frac{[HbO_2]}{[Hb]+[HbO_2]} * 100\%, \qquad \textit{Equation 3}$$

where $HbO_2$ is the oxygenated haemoglobin and $Hb$ is the deoxygenated

haemoglobin. However, for determining the absolute content of oxygen in blood, the amount of haemoglobin present must be taken into account according to Equation 4:

$$Oxygen\ content = Hb * 1.34 * SaO_2 + 0.003 * PaO_2. \qquad Equation\ 4$$

where the constant *1.34* is the oxygen carrying capacity (mL) per gram of haemoglobin (at 1 atmosphere), *Hb* is the concentration of haemoglobin in blood (g/dL), $SaO_2$ is the haemoglobin oxygen saturation (%, see Equation 3), the constant *0.003* represents the amount of oxygen dissolved in plasma (mL $O_2$/dL plasma/mmHg $PaO_2$) and $PaO_2$ is the partial pressure of oxygen (mmHg). Oxygen content is, therefore, expressed in units of mL $O_2$/dL blood.

As evident by Equation 4, the dissolved oxygen contributes minimally to the oxygen content under physiologic conditions. Thus, the total oxygen content is determined predominantly by the haemoglobin content and the oxygen saturation (but oxygen saturation is indeed dependent on the dissolved oxygen, as explained above), and related linearly to both variables (Guyton and Hall, 2000).

However, it must be kept in mind that these definitions simplify the complex matter of oxygen transport in blood since other forms of haemoglobin are found in blood and haemoglobin can also carry other molecules than oxygen and then referred to as dyshaemoglobin. The most relevant dyshaemoglobin for the purpose of this thesis is carboxy-haemoglobin, which is a stable compound of normal haemoglobin molecule combined with carbon monoxide instead of oxygen. Haemoglobin has indeed much higher affinity for carbon monoxide than oxygen and this stable carboxyhaemoglobin prevents oxygen from binding with haemoglobin. The body produces small amounts of carbon monoxide naturally but the fraction of carboxyhaemoglobin is normally less than 2% in humans, although this fraction can be up to 8% in current smokers (Fischbach and Dunning III, 2008). Since carboxyhaemoglobin has very similar colour as oxygenated haemoglobin it can affect the measurements of oxygen saturation. Therefore, it is important to know the smoking habits of patients undergoing retinal oximetry.

### 1.3.2 Principles of retinal oximetry

The retinal arterioles and venules can be seen directly through lenses and differentiated simply according to their colour, as oxygenated and deoxygenated haemoglobin have different colours. Retinal arterioles will have a light red tone, whereas the venules appear more dark red. Retinal oximetry is a non-invasive method that uses the different colour of haemoglobin at diverse oxygen levels for the measurement of oxygen saturation of haemoglobin in retinal arterioles and venules. This can be accomplished since light absorptivity of oxygenated and deoxygenated haemoglobin is different at most wavelengths of light but identical at some wavelengths, as can be seen in **Figure 5**.

The wavelengths where light absorptivities of oxygenated and deoxygenated haemoglobin are the same, like at 570 nm, are called isosbestic wavelengths and at those wavelengths light absorptivity is not sensitive to difference in oxygen saturation. On the contrary, at all other non-isosbestic wavelengths, such as at 600 nm, light absorptivity is sensitive to different oxygen saturation levels.

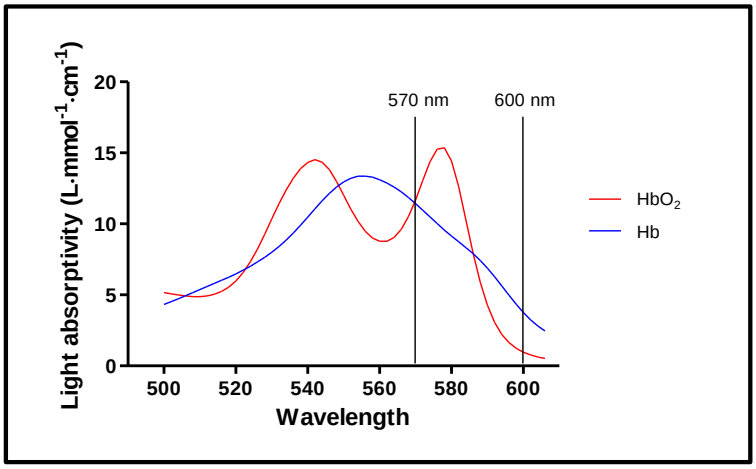


**Figure 5.** Light absorbance of oxygenated haemoglobin ($HbO_2$) and deoxygenated haemoglobin (Hb) at different wavelengths. Light absorbance is a measure of how much light a solution absorbs per unit concentration of the solution and per unit path length through the solution. Image prepared from data in Zijlstra et al. (2000).

In dual-wavelength retinal oximetry, two oximetry images are simultaneously captured at two different wavelengths, one isosbestic and one non-isosbestic wavelength. The same measurement points are selected on the oximetry images at the two different wavelengths and light intensity, or brightness value of pixels, is measured on the selected vessels (I) and to the side of the vessels ($I_0$), see **Figure 6**. The blood inside the vessels influences the brightness of the vessel but not the brightness to the side of the vessel.

The light absorbance of oxygenated and deoxygenated haemoglobin at different wavelengths ($\lambda$) can also be described with the optical density at respective wavelengths ($OD_\lambda$):

$$OD_\lambda = log(\frac{I_0}{I}). \qquad \textit{Equation 5}$$

Equation 5 shows that greater light absorbance results in higher optical density. Optical density of a blood vessel at isosbestic wavelength (570 nm) is not dependent on oxygen saturation but solely on vessel diameter and factors other than oxygen saturation, whereas at a non-isosbestic wavelength (600 nm) the optical density will also be sensitive to oxygen saturation.

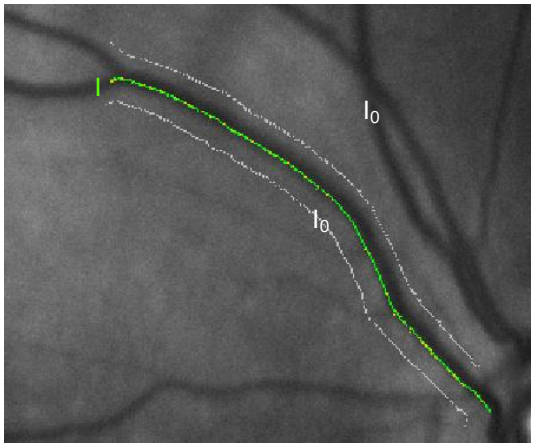


**Figure 6.** Monochrome oximetry image showing how measurements of light absorbance on a specific retinal vessel are performed. The green (570 nm) / yellow (600 nm) overlay shows where the light intensity on a vessel is measured (I). The white overlay is the light intensity of the background (I0). Courtesy of J. Valgerdur Kristjansdottir.

The ratio of optical densities (*ODR*) can be calculated from the optical density values at the two different wavelengths according to Equation 6:

$$ODR = \frac{OD_{600}}{OD_{570}}. \qquad \textit{Equation 6}$$

Optical density ratio from optical densities at non-isosbestic and isosbestic wavelengths is therefore sensitive to oxygen saturation, while the effects of other factors will for the most part have a tendency to cancel out. Moreover, the optical density ratio has been shown (Beach et al., 1999; Harris et al., 2003) to have an inverse and approximately linear relationship to oxygen saturation ($SaO_2$) as expressed in Equation 7:

$$SaO_2 = a * ODR + b, \qquad \textit{Equation 7}$$

where *a* and *b* are constants. The calibration of *a* and *b* for the retinal oximeter used in this thesis will be described in chapter 3.1.5.

### 1.3.3 Development of retinal oximetry

Until recently, most of the data on ocular oxygenation had come from invasive studies on animals or humans undergoing surgery. A reliable non-invasive retinal oximeter is therefore very important, as it enables the analysis of retinal oxygenation in humans in a safe and efficient manner.

The development of non-invasive retinal oximeters began more than half a century ago with the pioneering work of Hickam et al (1959), who were the first to demonstrate that a linear relationship was found between retinal vessel

oxygen saturation and a ratio of vessel optical densities at two different wavelengths, where one of the wavelengths was insensitive to change in oxygen saturation and the other sensitive to this change. They achieved this by using special filters and photographic films to obtain fundus images with two wavelengths of light, from which they could estimate the light intensities and calculate the retinal vessel oxygen saturation in humans. They even demonstrated that their instruments were sensitive to changes in oxygen saturation by altering the proportion of oxygen in the inhaled air and measuring the effect on retinal oxygen saturation and retinal vascular reactivity. Moreover, in addition to their normal population, they also had patients with vascular disorders, such as hypertension and diabetes, who were found to have significantly reduced constrictor response of the retinal arterioles (Hickam et al., 1959).

This innovative work of Hickam et al. was continued by Laing et al. (1975), who also developed retinal oximetry technique with two wavelengths that could be used to measure oxygen saturation in retinal vessels in rabbits and was shown to be sensitive to changes in retinal arteriolar saturation.

While the development of the dual-wavelength retinal oximetry continued, other research groups emerged with multiwavelength technologies and made important contributions to the development of the retinal oximetry technique. Delori et al. built a retinal oximeter that could scan retinal vessels in real-time with three wavelengths, thereby compensating for the effects of light scattering, while measuring optical density of each vessel. Their oximeter could only scan a small area of the retina at a time but it did include a vessel tracker to reduce the effects of eye movements during scanning (Delori, 1988; Sebag et al., 1989). Later, Denninghoff et al. developed another retinal oximetry method based on scanning but using four wavelengths. They conducted several studies on pigs evaluating the effects of blood loss (Denninghoff et al., 1997, 1998, 2003) and different light paths (Smith et al., 2000) on oximetry results. At a similar time, Schweitzer et al. (1995, 1999, 2001) made a retinal oximeter from an imaging spectrograph coupled to a fundus camera allowing simultaneous measurements of oxygen saturation and vessel diameter of retinal vessels using multiple wavelengths. This technique was used to develop a model for the calculation of absolute oxygen saturation from oxygenated blood in glass cuvettes (Schweitzer et al., 2001). However, only a small slit of 1.5 mm x 40 µm from the fundus could be measured at a time.

Concurrently, Beach and colleagues presented their work using dual-wavelength technique (Beach et al., 1999; Tiedeman et al., 1998) based on similar principles as Hickam and associates had used in their innovative experiments forty years earlier (Hickam et al., 1959). However, digital imaging systems, computer technologies and optical solutions were now available. Their technique was based on a modified fundus camera that was attached to an image splitter and a digital camera, which made it possible to simultaneously

capture two images at two separate wavelengths (569 and 600 nm), thereby eliminating the problem of eye movements from some of the earlier methods. The sensitivity of their method was tested with different proportions of oxygen inhaled, and they also made an attempt to correct for artificial effects of vessel diameter and fundus pigmentation (Beach et al., 1999).

Crittin et al. (2002) used a similar optical approach as Beach et al. in developing their dual-wavelength oximetry system. A few years later Hammer et al. (2008) developed a distinctive dual-wavelength retinal oximeter using a different optical approach and other wavelengths (548 and 610 nm), referred to as the Imedos system. This oximeter has now been used in several studies on retinal oximetry in healthy persons (Hammer et al., 2011; Man et al., 2014) as well as patients with various diseases, such as diabetes mellitus (Hammer et al., 2009a; Man et al., 2015), retinal arterial occlusion (Gehlert et al., 2010; Hammer et al., 2009b), glaucoma (Türksever et al., 2015), retinitis pigmentosa (Türksever et al., 2014a), chronic obstructive pulmonary disease (Palkovits et al., 2013) and giant cell arteritis (Türksever et al., 2014b).

Other research groups using diverse approaches have also contributed to the progress in retinal oximetry. Ramella-Roman et al. (2008) introduced a multiaperture camera system for the measurement of retinal oxygen saturation. Li et al. (2011) used adaptive optics confocal scanning laser ophthalmoscope to measure oxygen saturation in small retinal vessels. Kagemann et al. (2007) developed spectral retinal oximetry using Fourier domain optical coherence tomography. Khoobehi et al. (2004, 2011) produced a hyperspectral system to measure oxygen saturation in vessels of the optic nerve head (tested in monkeys). Harvey and colleagues developed another hyperspectral instrument for measuring retinal oxygen saturation *in vivo* and in a model eye (Harvey et al., 2005; Mordant et al., 2011a, 2011b). Johnson and associates developed different hyperspectral system for measuring oxygen saturation within retinal vessels (Johnson et al., 2007; Kashani et al., 2011). Furukawa et al. (2012) used multicomponent analysis for determination of oxygen saturation of retinal capillaries. Kristjansdottir et al. (2014) mesured retinal vessel oxygen saturation using scanning laser ophthalmoscopy with dual-wavelength oximetry algorithm. For more extensive review on development on retinal oximetry see Harris et al. (2003), Hardarson (2013) and Beach (2014).

### 1.3.4 Retinal oximetry in healthy individuals

In the last couple of decades, digital cameras and computer technology have developed tremendously and modern spectrophotometric retinal oximetry has benefitted greatly from these advancements. Retinal oximeters have now been shown to be sensitive and reliable for measurements in healthy individuals with good repeatability and reproducibility (Blondal et al., 2011; Hardarson et al., 2006; Jani et al., 2014; Lasta et al., 2012; Man et al., 2013, 2015; O'Connell et al., 2014; Schweitzer et al., 1999; Shahidi et al., 2013;

Türksever et al., 2015; Yip et al., 2014). Moreover, retinal oximeters have demonstrated changes in retinal oxygen metabolism with alterations in illumination (Hardarson et al., 2009a), flash intensities (Heitmar and Cubbidge, 2013) and by flicker light stimulation (Hammer et al., 2011).

The majority of the aforementioned studies on healthy individuals have been conducted to test the sensitivity and reliability of the retinal oximeters, the others to determine physiological changes in the healthy retina subjective to stimulation. Very recently, however, two separate studies presented normative data on healthy subjects (Jani et al., 2014; Man et al., 2014), although one of the studies included only young adults (Man et al., 2014).

Jani et al. (2014) measured retinal oxygen saturation using the Oxymap T1 oximeter and version 2.3.1 of the Oxymap Analyser software but version 2.2.1 was used for the studies in this thesis. The study by Jani et al. included 61 healthy persons of multi-ethnic origin ranging between 19-74 years of age, mean age 44±15 years. Mean arterial oxygen saturation of the right eyes for the entire group was 90.4±4.3% and mean venular oxygen saturation was 55.3±7.1%. No difference was found between genders or between the various ethnic groups, or specifically in relation to different iris pigmentation. Their main conclusion was that increasing age was found to be significantly associated with decreased venular and arteriolar oxygen saturation as well as increase in arteriovenous difference (Jani et al., 2014).

The study by Man et al. (2014) was performed using the Imedos system and included 50 healthy persons aged 18-58 years old, though most subjects were younger than 40 years and the median age 26 years. Median arterial oxygen saturation of the right eyes for the entire group was 95.94% (range 91.53% to 98.49%) and median venular oxygen saturation was 62.35% (range 57.65% to 64.17%). In all their analyses, both eyes from each person were used. In contrast to Jani et al. (2014), this study of young healthy individuals by Man et al. (2014) showed an increase in arteriolar and venular oxygen saturation with increasing age. No significant association was found between age and arteriovenous difference, while systemic oxygen saturation from finger pulse oximeter was positively correlated with greater arteriovenous difference.

### 1.3.5 Retinal oximetry in diseases

Retinal oximetry has also demonstrated significant changes in retinal vessel oxygenation in several eye disorders, including diabetic retinopathy (Hammer et al., 2009a; Hardarson and Stefánsson, 2012a; Jørgensen and Bek, 2014; Jørgensen et al., 2014; Khoobehi et al., 2013; Kashani et al., 2014; Man et al., 2015; Schweitzer et al., 2007), retinal vein occlusions (Eliasdottir et al., 2015; Hardarson and Stefánsson, 2010, 2012b), retinal arterial occlusions (Hardarson et al., 2013a), retinitis pigmentosa (Eysteinsson et al., 2014; Türksever et al., 2014a; Ueda-Consolvo et al., 2015) and glaucoma (Goharian et al., 2015;

Michelson and Scibor, 2006; Olafsdottir et al., 2011, 2014; Vandewalle et al., 2014). This technology has also showed variations in retinal oxygenation in response to pharmocological and surgical interventions for glaucoma (Hardarson et al., 2009b; Siesky et al., 2008, 2010; Traustason et al., 2009) and central retinal vein occlusions (Traustason et al., 2014) as well as after vitrectomy (Lim et al., 2014; Šín et al., 2014). Preliminary data on oximetry in patients with giant cell arteritis show significant alterations in retinal vessel oxygen saturation despite no visible ophthalmic pathology related to giant cell arteritis (Türksever et al., 2014b). Moreover, a recent study on oximetry in patients with Alzheimer's disease reported significant higher mean values of retinal vessel oxygen saturation in both arterioles and venules in comparison to a healthy cohort (Einarsdottir et al., 2015). Retinal oximetry can reliably measure the systemic hypoxia in Eisenmenger syndrome (Traustason et al., 2011) as well as the changes in retinal oxygen saturation with and without systemic oxygen therapy in patients with severe form of chronic obstructive pulmonary disease (Palkovits et al., 2013).

Most of these studies on retinal oximetry in health and diseases in the last decade were performed with another type of oximeter or an older prototype of the oximeter used in this thesis. In the present studies, Oxymap T1 retinal oximeter was used. It is a non-invasive dual-wavelength spectrophotometric retinal oximeter built and developed by members of the Icelandic retinal oximetry research group. The Oxymap T1 retinal oximeter is connected to specialised Oxymap Analyser software, which calculates haemoglobin oxygen saturation based on the same principle as the earlier work by Hickam et al. (1959) and Beach et al. (1999). The oximeter and the software will be described in detail in chapters 3.1.3 to 3.1.6.

## 1.4 Age-related macular degeneration (AMD)

Age-related macular degeneration (AMD) is a bilateral degenerative disease affecting the macula. The macular area encompasses less than ten percent of the entire retina, but is nevertheless responsible for the most useful vision, i.e. the sharp central vision. AMD lesions developing in the macula can therefore have a major impact on visual function and AMD is the leading cause of irreversible blindness in the elderly in industrialised nations (Congdon et al., 2004).

AMD causes progressive degeneration of the retina, with the retinal pigment epithelium at the centre of the pathogenesis. The retinal pigment epithelium is a monolayer of pigmented cells joined by tight junctional complexes, thereby forming the important outer blood-retinal barrier that protects the neural retina. The retinal pigment epithelium contributes to the retinal function in several ways, such as by absorbing light, phagocytosing photoreceptor outer segments and by participating in the retinal metabolism (Schubert, 2012). Therefore, the vitality of the retinal pigment epithelium is fundamental to retinal health, in particular the photoreceptors.

The retinal pigment epithelium is separated from the underlying choriocapillaris by Bruch's membrane. Bruch's membrane consists of five layers, with the basement membrane of the retinal pigment epithelium as the innermost layer and then the inner collagenous zone, a central band of elastic fibers, the outer collagenous zone and finally the basement membrane of the choriocapillaris as the outermost layer (Schubert, 2012). One of the primary functions of Bruch's membrane is regulation of diffusion of biomolecules between retinal pigment epithelium and choroid. The diffusion is mainly by passive means but it is influenced by several factors, such as age. With increased age Bruch's membrane is known to thicken and calcify, thereby slowing the transport of water, oxygen and other metabolites (for review see Booij et al., 2010). In AMD, additional structural changes in Bruch's membrane are seen, which cause even more disturbances in normal diffusion (Booij et al., 2010).

The choriocapillaris is the innermost layer of the choroid, consisting of lobular capillary bed that lies in a single plane beneath Bruch's membrane. The choriocapillaris is also suspected to play a role in the pathogenesis of AMD, as the decrease in its density and diameters of the capillaries seen with normal aging, is even greater in patients with AMD (McLeod et al., 2002; Ramrattan et al., 1994).

Although Bruch's membrane and the choroid are essential for the retinal microenvironment and play a role in the pathogenesis of AMD, dysfunction and degeneration in the retinal pigment epithelium are believed to be the critical event in the pathogenesis of AMD (Ambati and Fowler, 2012). Although numerous studies have contributed to the understanding of development and advancement in AMD, the complete picture is still elusive, see further chapter 1.6.

## 1.5 Classification of AMD

The clinical classification of AMD is based on stages according to progressive changes in the macula. Through the years, various classification schemes have been described (Age-Related Eye Disease Study Research Group, 1999; Bird et al., 1995; Davis et al., 2005; Ferris et al., 2005; Klein et al., 1991) but recently a large group of experts agreed upon an update of the classification schemes in order to increase consistency in the nomenclature of AMD (Ferris et al., 2013). The main stages of AMD are early AMD (chapter 1.5.1), intermediate AMD (chapter 1.5.2) and late AMD (chapter 1.5.3).

### 1.5.1 Early AMD

The hallmark of early AMD is drusen (**Figure 7**) (Ferris et al., 2013). Drusen appear as yellow spots in the retina and consist of accumulations of insoluble, extracellular debris that builds up under the retina, usually between the retinal pigment epithelium and Bruch's membrane (**Figure 9**). Drusen can also form internally to the retinal pigment epithelium and those are referred to as reticular drusen.

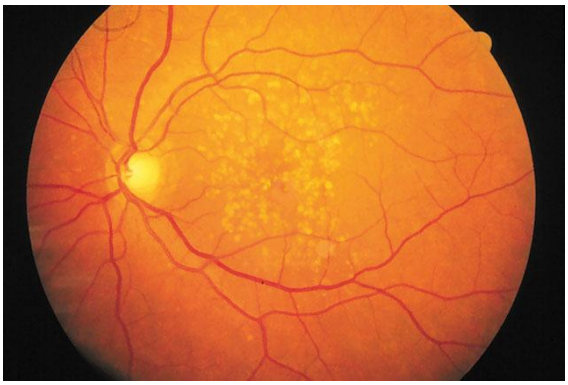

**Figure 7.** Fundus with early AMD showing drusen on the macula. Courtesy of Professor Fridbert Jonasson.

Drusen can be classified into hard and soft. The hard drusen are small and have discrete margins, whereas the soft drusen are larger with indistinct edges. All the classification systems subdivide drusen into defined categories based on their appearance, number and size (Age-Related Eye Disease Study Research Group, 1999; Davis et al., 2005; Ferris et al., 2005; Klein et al., 1991). In short, according to the new clinical classification scheme based on the Age-Related Eye Disease Study (AREDS) database (Ferris et al., 2013), persons with small hard drusen (<63 µm) are considered to have normal aging changes and not AMD, whereas persons with medium sized drusen (≥63 to <125 µm) and no signs of pigmentary abnormalities (hyper- or hypopigmentation) related to AMD, are classified as having early AMD. Pigment changes together with medium sized drusen (in one or both eyes) increase the risk of disease progression substantially (Ferris et al., 2013).

### 1.5.2 Intermediate AMD

Patients with large drusen (>125 µm) and/or the presence of pigmentary abnormalities in the macula associated with at least one medium sized or large drusen, are classified as having intermediate AMD (Ferris et al., 2013). These pigmentary abnormalities can be either hyper- or hypopigmentation, and are believed to be attributable to degenerative changes in the retinal pigment epithelium cells, causing mobilization of their high content of melanin (Okubo et al., 1999). The degeneration of the retinal pigment epithelium, however, also causes concomitant degeneration of overlying photoreceptors since viable retinal pigment epithelium is fundamental for normal photoreceptor cell metabolism.

### 1.5.3 Late AMD

#### *1.5.3.1 Geographic atrophy*

Geographic atrophy is the dry advanced form of AMD. It is also referred to as the non-exudative AMD since a prerequisite for the definition of pure

geographic atrophy is the absence of choroidal neovascularisation. Geographic atrophy and choroidal neovascularisation can, however, occur simultaneously in the same eye. Geographic atrophy or the clinical appearance of geographic atrophy can also form after regression of neovascular AMD (Sunness et al., 1999). Moreover, treatment of neovascular AMD (see chapter 1.5.3.2) with antibodies against vascular endothelial growth factors (anti-VEGF; see chapter 1.8) may even trigger the development of atrophic lesions of the retinal pigment epithelium that mimic geographic atrophy (Lois et al., 2013; Tanaka et al., 2015).

Geographic atrophy is characterised by sharply delineated relatively round area of atrophy with apparent loss of the retinal pigment epithelium, which makes choroidal vessels more easily seen than in surrounding areas (**Figure 8**).

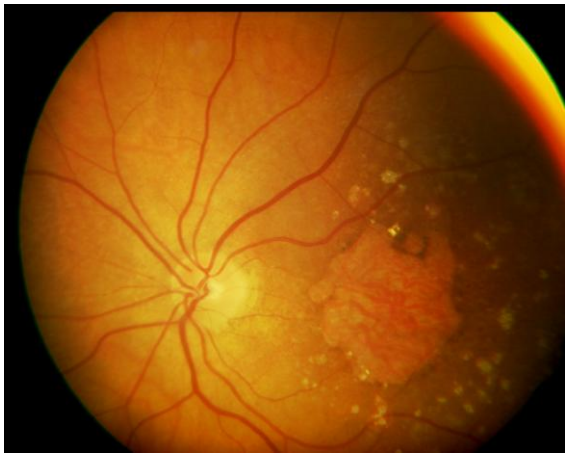

**Figure 8.** Fundus with geographic atrophy in the macula. This characteristic geographic atrophy lesion shows the distinctive sharp borders of atrophy and clearly visible choroidal vessels below the atrophic retina. Courtesy of Clinical Associate Professor Haraldur Sigurdsson.

According to the international classification scheme by Bird et al. (1995), the area of atrophy must be at least 175 µm in diameter for it to be defined as geographic atrophy. The development of geographic atrophy lesions is commonly characterised by a sequential progression from large drusen to hyperpigmentation, which is followed by regression of drusen and appearance of hypopigmentation that ultimately develops into atrophic areas (Klein M. L. et al., 2008). Geographic atrophy is commonly found bilaterally and progression is often a symmetrical process, although the size of the atrophic area may differ substantially between the eyes (Fleckenstein et al., 2010; Sunness et al., 2007).

Histopathologically, geographic atrophy involves a loss of the retinal pigment epithelium cell layer, accompanied by death of the overlying retinal

photoreceptors (Solomon and Sunness, 2008) as well as deterioration and reduction in density of the underlying choriocapillaris and choroidal vessels (McLeod et al., 2002), see **Figure 9**. Geographic atrophy is commonly slowly progressive with development of one or several small parafoveal lesions, which over time increase in size and coalesce, often sparing the central fovea, and thereby the visual acuity to some extent, until late in the disease course (Sarks et al., 1988).

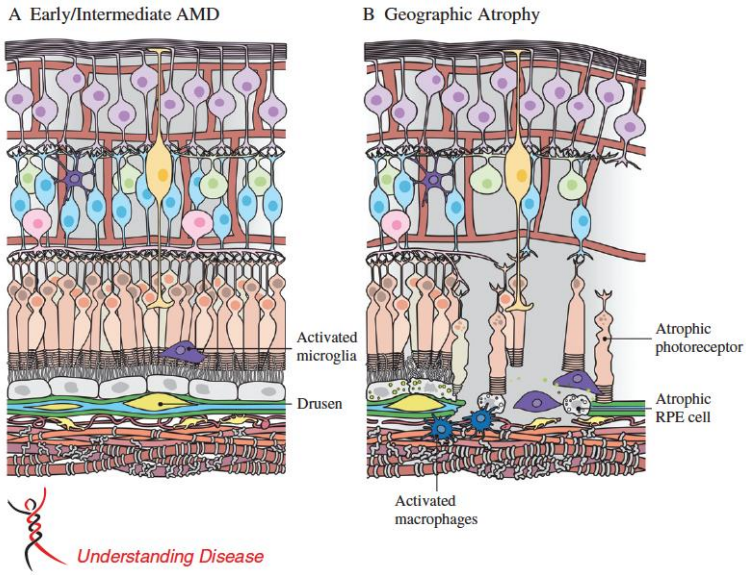


**Figure 9.** Schematic diagrams of non-neovascular AMD. (A) Early/intermediate AMD with drusen accumulating under the retinal pigment epithelium and within the Bruch's memrane. Activated microglia suggest that inflammation is present in the outer retina. (B) Geographic atrophy with severely disrupted Bruch's membrane and pronounced atrophy of retinal pigment epithelium and photoreceptors as well as other retinal cells, which are dependent on the underlying retinal pigment epithelium. The more numerous activated microglia and macrophages in the atrophic area suggest increased inflammation in more advanced disease. Reprinted from J Pathol, 232(2), van Lookeren Campagne et al., Mechanisms of age-related macular degeneration and therapeutic opportunities, 151-164, ©2014, with permission of Genentech and John Wiley & Sons Ltd.

### *1.5.3.2 Neovascular AMD*

Neovascular AMD is the wet advanced form of AMD, also known as exudative AMD. In the previously mentioned update of the classification nomenclature of AMD by Ferris et al. (2013), neovascular AMD was suggested as the preferred term for the wet form of late AMD and will therefore be used in this thesis (even though the term exudative AMD was used in Papers III and IV).

Neovascular AMD is characterised by an abnormal growth of immature blood vessels derived from the choroid. These new vessels, most often

localised in the macular area, break through the Bruch's membrane in their proliferation towards or into the retina. This process is called choroidal neovascularisation and can be subdivided into two main forms according to their appearance on fluorescein angiography, the occult and classic form (see **Figure 10**), but a mixture of these two forms can also occur. The occult form consists of abnormal vessels that grow through Bruch´s membrane but not through the retinal pigment epithelium, and thus cause subretinal fluid accumulation. In the classic form, the new abnormal choroidal vessels grow through Bruch´s membrane as in the occult form, but in the classic form they also penetrate the retinal pigment epithelium and may thus, cause both subretinal and intraretinal fluid accumulation (**Figure 10**).

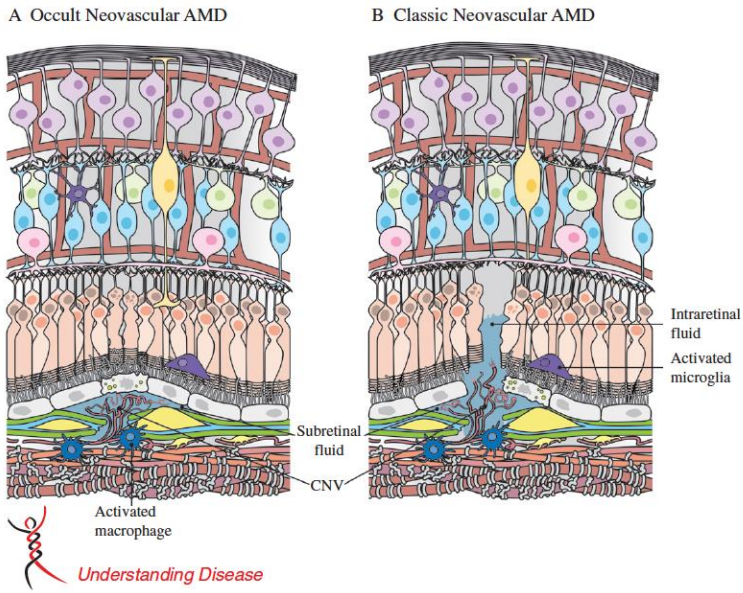


**Figure 10.** Schematic diagrams of neovascular AMD. (A) Occult neovascular AMD with choroidal neovascularisation (CNV) disrupting the Bruch's membrane, allowing fluid from the leaky vessels to accumulate under the retinal pigment epithelium, which is intact. (B) Classic neovascular AMD with choroidal neovascularisation disrupting both the Bruch's membrane and the retinal pigment epithelium, causing sub- and intraretinal fluid accumlation. In both types of neovascular AMD, the activated macrophages and microglia suggest that inflammation is present. Reprinted from J Pathol, 232(2), van Lookeren Campagne et al., Mechanisms of age-related macular degeneration and therapeutic opportunities, 151-164, ©2014, with permission of Genentech and John Wiley & Sons Ltd.

Neovascular AMD involves leakage of blood (**Figure 11**), fluid and lipids into the subretinal space or the retina (intraretinal), which may cause hemorrhagic or serous detachment of the retina (Bird et al., 1995). These pathological processes can abruptly threaten the central vision, and if untreated cause scarring and permanent visual acuity loss.

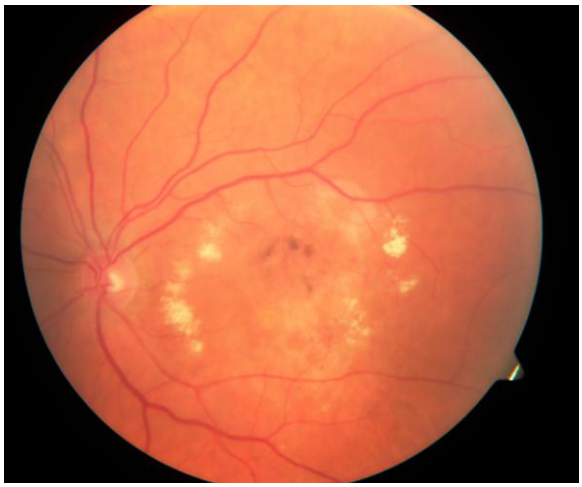

**Figure 11.** Fundus with neovascular AMD showing macular hemorrhage and suspected subretinal fluid with surrounding exudates. Courtesy of Professor Fridbert Jonasson.

## 1.6 Pathophysiology of AMD

The pathophysiology of AMD has been studied extensively and several different mechanisms are believed to contribute to the disease at various stages of its development, though the exact mechanism is not yet known. A variety of hypotheses has been proposed and most suggest that retinal pigment epithelium dysfunction in the macula is the critical event in the pathogenesis of AMD (Ambati and Fowler, 2012). However, the hypotheses differ when it comes the cause of the retinal pigment epithelium dysfunction. Genetic factors, environmental factors as well as physiological factors have all been proposed. Genetic studies have suggested inflammatory components (chapter 1.6.1), clinical trials have indicated oxidative stress (chapter 1.6.2), while physiological studies have pointed towards ischaemia (chapter 1.6.3). As the name of the disease implies, the greatest risk factor for initiation of AMD is increasing age (Jonasson et al., 2011; Smith et al., 2001). More importantly, increased age also causes increased progression of AMD (Chew et al., 2014). Previously, our research group studied AMD in the very old with family history of the disease as part of the Icelandic Genetics Study on AMD. This study included 897 AMD patients aged 75 years and older, thereof eight centenarians. The results showed that the proportion of patients with late AMD, in comparison to early AMD, increased successively with increasing age, and late AMD was found in all the centenarians (Geirsdottir et al., 2007).

### 1.6.1 Genetics

Although many factors are involved in the risk and progression of AMD, it is fully acknowledged that there is a strong genetic contribution. Previously, it has been

estimated that genetic risk factors can explain up to 71% of the variation in the overall severity of AMD (Seddon et al., 2005). Studies on twins have found concordance rates of AMD to be significantly higher between monozygotic twins than dizygotic twins (Klein M. L. et al., 1994; Meyers, 1994). Familial aggregation studies have found evidence of significant correlations of AMD between first-degree relatives (Klaver et al., 1998; Seddon et al., 1997; Shahid et al., 2012). However, AMD has indeed been shown to be genetically heterogeneous in nature and genetic linkage analyses of ancestries from AMD patients have revealed multiple candidate loci within most chromosomes. Recently, our research group, as part of the AMD Gene Consortium, published the results of the largest analysis to date of genetic risk in AMD (Fritsche et al., 2013). This analysis included over 17 000 AMD cases and more than 60 000 controls. The results confirmed many of the previously identified loci but interestingly seven new loci were also identified. These loci are parts of diverse biological and molecular pathways, such as pathways regulating angiogenesis, the complement system, lipid metabolism and extracellular matrix biology, all of which are believed to be vital for the health of the retina and macula in particular (Zhang et al., 2012). Although these new loci do not add as much to the estimated heritability in AMD as the previously confirmed loci, they do add a few more pieces to the puzzle in the complex picture of genetics in AMD. The two strongest loci for AMD risk identified so far are the *CFH*, located at 1q31.3, and *ARMS2/ HTRA1*, located at 10q26 (Fritsche et al., 2013). A decade ago several genome-wide association studies simultaneously confirmed an amino acid-changing variant in the *CFH* gene (Edwards et al., 2005; Haines et al., 2005; Klein R. J. et al., 2005) and the *ARMS2/HTRA1* locus (Jakobsdottir et al., 2005; Rivera et al., 2005). Moreover, the results from the Icelandic Genetic Study on AMD confirmed that a variant of *CFH* had significant association with late AMD, and even more importantly showed that *CFH* is a major risk factor of soft drusen, concluding that additional factors (genetic and/or environmental) might be required for progression to late AMD (Magnusson et al., 2006). Recently, Klein et al. (2013a) conducted a study on the long-term risk of incidence and progression of AMD in relation to age and these two genes with the most attributable risk of AMD, *CFH* and *ARMS2*. The results indicated that when early AMD is present, then knowledge of the phenotype is more effective in risk assessment than knowledge of genetic risk from these two AMD genes.

The quest for determination of the functionality of the disease loci is currently ongoing but one variant in the *CFH* gene has been shown to impair the binding of *CFH* to Bruch's membrane, resulting in overactivation of the complement pathway (Clark et al., 2010), thereby triggering the immune system. The function or causality of variants within the *ARMS2/HTRA1* has been more difficult to establish (Yang et al., 2010), especially since the most significantly associated haplotype extends across both genes, making it even more challenging to assign causality to each specific variant (Hadley et al.,

2010). One study, however, has shown that *ARMS2* is expressed in the retinal pigment epithelium cells and suggests it might play a role in their phagocytosis function and thereby, possibly participate in the development of AMD (Xu et al., 2012). For further information on chromosome 10q26 locus see recent review by Wang (2014) as well as chapter 1.6.2 for the relationship between genetic susceptibility and oxidative damage.

Multiple variants in other genes in the complement pathway have been identified, including in *C3* (Park et al., 2009; Yates et al., 2007). Recently, our research group identified a rare nonsynonymous single nucleotide polymorphism K155Q in the *C3* gene (Helgason et al., 2013). This variant is significantly associated with AMD and fully independent from the earlier reported common single nucleotide polymorphisms in *C3* found in association with AMD (Park et al., 2009; Yates et al., 2007).

One gene of particular interest to researchers is the *VEGFA* but the main treatment of neovascular AMD is with antibodies against VEGF that block all VEGF-A isoforms (see further chapter 1.8). *VEGFA* is an endothelial cell-specific mitogen with various effects, including increasing vascular permeability and inducing angiogenesis and vasculogenesis. In 2006, a locus in the promoter region of *VEGFA* was identified and associated with AMD (Haines et al., 2006). More recently, a common variant related to the *VEGFA* gene was identified and confirmed as being associated with advanced AMD, possibly by causing dysregulation in angiogenesis (Fritsche et al., 2013; Yu et al., 2011).

Although genetic susceptibility is undoubtedly an essential part in the pathogenesis of AMD, it is important to note that none of these risk alleles is believed to be entirely causal, since all have been found in the control populations as well. However, hereditary predisposition can perhaps be thought of as a pathological driver in affected persons, even by causing damage in the beginning of the disease process, and thereby increasing susceptibility to other risk factors, such as oxidative stress (see further in chapther 1.6.2). Several genetic variations associated with AMD susceptibility have provided evidence for the role of oxidative stress in the pathogenesis of AMD. Of particular interest are the previously mentioned loci *ARMS2/HTRA1* and *CFH*, which have also been shown to be associated with smoking. Moreover, the combination of *ARMS2/HTRA1* polymorphism and smoking results in higher risk for AMD than the sum of effects of either factor alone (Schmidt et al., 2006). The same is true for the combination of *CFH* polymorphism and smoking (Delcourt et al., 2011). Therefore, it is likely that there is some interaction between the immune system and oxidative processes.

### 1.6.2 Oxidative damage

The retina, in particular the macula, consumes oxygen at a rate, which is among the highest of all tissues (per unit weight). The retina is, therefore,

subject to extensive generation of reactive oxygen species, such as free radicals, hydrogen peroxide and singlet oxygen, which can cause oxidative damage in the retina. However, oxidative damage is generally minimized by the existence of a variety of antioxidants and repair systems. Unfortunately, with increasing age the antioxidant capacity usually decreases and the reparative systems become less efficient, causing more oxidative damage in the retina (for review see Jarrett and Boulton, 2012).

Reactive oxygen species can be derived from numerous endogenous and exogenous sources. The most common endogenous sources are within the cells, i.e. mitochondria, enzyme systems and photosensitizers, whereas the most common exogenous causes are the general light exposure as well as cigarette smoking but smoking has been shown to be the second most consistent risk factor of AMD, after age (Klein et al., 1993; Smith et al., 1996; Vingerling et al., 1996); for a systematic review see Chakravarthy et al. (2010). Cigarette smoke is known to contain a high number of toxic compounds, which exert their pathological effects through different biochemical pathways. Drusen themselves may also contribute to oxidative damage in AMD, but amyloid-β, which has been found in both drusen and retinal pigment epithelium cells, has been shown to increase formation of reactive oxygen species as well as reduce the antioxidant capacity of retinal pigment epithelium cells (Bruban et al., 2009).

Consequently, increased oxidative damage results in increased retinal dysfunction and gradual retinal cell loss, which, in combination with genetic susceptibility and possibly other retinal modifiers, could cause a progression to the severe pathology associated with late AMD (Jarrett and Boulton, 2012).

### 1.6.3 Ischaemia and hypoxia

In addition to the aforementioned factors in the pathogenesis of AMD, ischaemia has been proposed to play a major role in the development of AMD, specifically neovascular AMD (Ciulla et al., 2001; Feigl et al., 2007; Friedman et al., 1995; Grunwald et al., 1998, 2005; Pauleikhoff et al., 1990). Ischaemia is inadequate blood flow to provide sufficient oxygenation in tissues, which can thereby lead to tissue hypoxia as well as insufficient removal of metabolites (Guyton and Hall, 2000). For further information on ischaemia in AMD, see reviews by Stefánsson et al. (2011) and Feigl (2009).

Already in 1937, Verhoeff and Grossman (1937) proposed that the pathogenesis of AMD could be attributed to impairment of choroidal blood flow as well as the development of drusen. In 1963, Friedman et al. (1963) showed that drusen are manifestations of an exudative process that involves the choriocapillaris and later suggested that impairment of choroidal perfusion is critical in the etiology of AMD (Friedman, 2008). Grunwald et al. (2005) has also demonstrated systematic decrease in choroidal circulatory parameters associated

with an increase in the severity of AMD, thereby suggesting a role for ischaemia in the development of choroidal neovascularisation in neovascular AMD. Feigl (2009) points out that the high number of metabolically active photoreceptors and their post-receptoral pathways in the macula result in the particularly high oxygen demand in the macula compared to other areas of the retina, and hence the macular predilection to ischaemia in AMD.

The underlying cause of the choroidal ischaemia is not fully known but studies have pointed out the relationship between AMD and atherosclerosis (Friedman, 2000; Machalińska et al., 2012; Vingerling et al., 1995a). This vascular model of pathogenesis of AMD suggests that the deposition of lipids, which occurs in systemic arteries in atherosclerosis, also occurs in the sclera in AMD patients, thereby increasing the scleral rigidity in these patients (Friedman et al., 1989). This scleral rigidity can impair the choroidal blood flow through the sclera, and thus, decrease the clearance of lipoproteins from the retinal pigment epithelium. The lipoproteins, which are considered to be mostly waste products from the retinal metabolism (Dashti et al., 2006), then accumulate in the outer retina as well as in the retinal pigment epithelium and in the Bruch's membrane. These deposits successively cause atrophy of the retinal pigment epithelium and can even cause fractures in the Bruch's membrane, thereby allowing access for the choroidal vessels to sprout into the retina as choroidal neovascularisation (Friedman, 2000).

The data most strongly suggesting ischaemia in AMD are from measurements of choroidal blood flow, which are generally disturbed and resulting in choroidal ischaemia in these patients (Grunwald et al., 1998; Pauleikhoff et al., 1990). In the study by Grunwald et al. (1998), patients with non-exudative AMD were shown to have reduced choroidal blood volume and blood flow. The group extended that study and showed a decline in choroidal blood volume and blood flow with both increasing severity of AMD (Grunwald et al., 2005) and increasing extent of drusen (Berenberg et al., 2012). These studies do suggest the presence of choroidal ischaemia in AMD. Moreover, using indocyanine green angiography, Goldberg et al (1998) observed that choroidal neovascularisation in AMD is most commonly localized close to areas with poor choroidal perfusion. In a longitudinal study, Metelitsina et al. (2008) were able to demonstrate that development of choroidal neovascular membrane in AMD patients was preceded by decreased foveolar choroidal blood flow. Later, Boltz et al. (2010) extended upon these data with more homogenous study group, including only the fellow eye of patients with unilateral choroidal neovascularisation. Their results suggested that decreased choroidal perfusion was a risk factor for the development of choroidal neovascularisation in the fellow eye. Moreover, these longitudinal studies also suggested the role of choroidal hypoperfusion, and possibly choroidal ischaemia, in the pathogenesis of AMD.

Reduced choroidal blood flow may impair the retinal metabolism and lead to retinal hypoxia. Hypoxia is known to up-regulate angiogenic factors in the choroid, including vascular endothelial growth factor (VEGF) (Aiello et al., 1995). VEGF induces formation of the pathological vessels in choroidal neovascularisation, see further chapter 1.8. Hypoxia has been shown to cause upregulation of certain transcription factors named hypoxia-inducible factors, or HIFs (Gemenetzi and Lotery, 2014). Forsythe et al. (1996) showed that HIF-1 activate transcription of VEGF in hypoxic cells, but VEGF is the predominant growth factor stimulating choroidal neovascularisation in neovascular AMD (Schlingemann, 2004), as discussed more thoroughly in chapter 1.8. Moreover, HIF-1α and HIF-2α have specifically been shown to be present in choroidal neovascularisation membranes of AMD patients (Inoue et al., 2007; Sheridan et al., 2009).

Some of the cardinal features of AMD may also disturb the delivery of oxygen and nutrients from the choroidal vessels to the retina. Thickened Bruch's membrane, drusen and retinal edema can all increase the diffusion distance and may also change the diffusion coefficient (Stefánsson et al., 2011). As an example, the accumulation of large, confluent drusen increases the distance between the choroid and the retina, thereby decreasing the flow of oxygen from the choroid to the outer retina (Abdelsalam et al., 1999; Linsenmeier and Padnick-Silver, 2000).

Vitreoretinal adhesion has in recent years been suggested to contribute to the development of neovascular AMD. In 1996, Weber-Krause and Eckardt (1996) reported an association between AMD and the vitreomacular interface. Later, several research groups have shown that posterior vitreous detachment is significantly less common in patients with neovascular AMD compared with patients with non-neovascular AMD and controls (Krebs et al., 2007; Lee et al., 2009; Robison et al., 2009). Although none of these studies are conclusive, the data point to higher rates of vitreomacular adhesion and lower rates of posterior vitreous detachment in eyes with neovascular AMD compared with eyes with non-neovascular AMD or no AMD at all. Currently, there is, however, not sufficient evidence to determine whether vitreomacular adhesion promotes the development of neovascular AMD or if the reverse is true, that neovascular AMD promotes vitreomacular adhesion, for example through some of the local inflammatory mechanisms that possibly could strengthen the adhesion between the retinal inner limiting membrane and the posterior vitreous face. In our review on metabolic physiology in AMD, mechanisms for how vitreoretinal adhesion can contribute to neovascular AMD are further discussed (Stefánsson et al., 2011). As illustrated in **Figure 12**, vitreoretinal adhesion may decrease the transvitreal delivery of oxygen from areas with normal oxygen tension to hypoxic retinal areas because of the decreased diffusion coefficients in the viscous vitreous gel compared with the low viscosity fluids that accumulates behind the posterior vitreous detachment

(Gisladottir et al., 2009; Stefánsson et al., 1988b). Moreover, the viscous vitreous gel also leads to decrease in clearance of VEGF produced in hypoxic areas of the retina, thereby stimulating further choroidal neovascularisation. The vitreous has also been shown to consume a small amount of oxygen (Shui et al., 2009), which could possibly induce further hypoxia in retina.

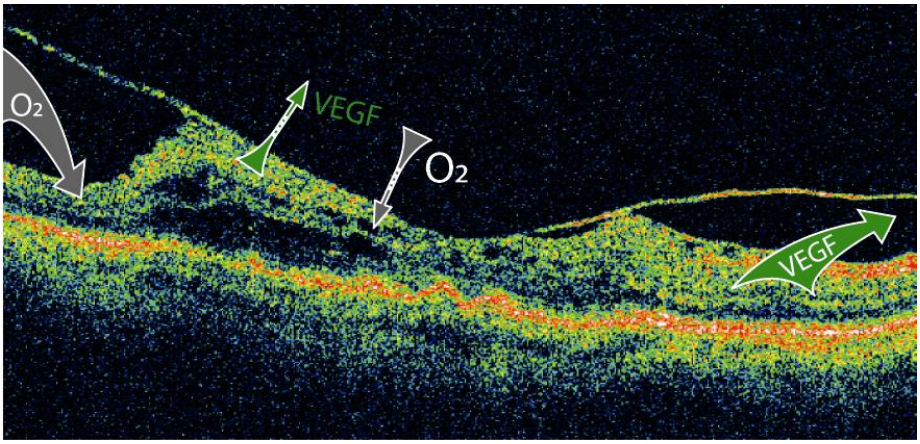


**Figure 12.** Optical coherence tomography (OCT) image of vitreoretinal adhesion. The arrows indicate the direction and magnitude of transport of oxygen ($O_2$) and vascular endothelial growth factor (VEGF) between the retina and vitreous where vitreoretinal adhesion is partially present (centre) as opposed to posterior vitreous detachment (to either side). Reprinted from Progr Ret Eye Res, 30(1), Stefánsson et al., Metabolic physiology in age-related macular degeneration, 72-80, ©2011, with permission from Elsevier.

It is likely that each of these aforementioned features of AMD may not by themselves create enough ischaemia and hypoxia to stimulate the development of neovascular AMD, but they may work together to do so.

## 1.7 Retinal circulation in AMD

### 1.7.1 Retinal blood flow in AMD

Only a few studies on blood flow in AMD patients have focused on the retinal blood flow. These studies are conducted with different techniques and give somewhat conflicting results. Sato et al. (2006) used Canon laser Doppler flowmeter and found no differences in retinal blood flow in major temporal arterioles among patients with late AMD (either neovascular AMD or geographic atrophy) in comparison with similarly aged healthy persons. However, increased pulsatility was seen with increased severity of the disease even though no change was seen in the mean blood flow over 2-seconds. Using a Heidelberg two-dimensional confocal scanning laser Doppler flowmeter, Remsch et al. (2000) found higher retinal capillary blood flow in the macula of patients with neovascular AMD compared to a age- and gender-matched healthy control group. However, these results could not be

replicated by Nowak et al. (2007) using comparable Heidelberg flowmeter. They found no difference in retinal capillary blood flow in the macula in neovascular AMD patients in comparison to a healthy age-matched control group. These studies on retinal blood flow in AMD patients are inconclusive and further studies are needed for determination if ischaemia is indeed present in the inner retina of AMD patients.

### 1.7.2 Retinal vessel oxygen saturation in AMD

Limited data can be found on retinal vessel oxygen saturation in patients with AMD. By evaluating reflectance spectra using spectroscopic techniques, Schweitzer et al. (2005) calculated retinal oxygen saturation in arterioles and venules in patients with early and late AMD as well as in their first generation descendants and healthy controls. Their results showed significantly reduced arteriovenous difference in patients with early AMD in comparison to healthy controls, patients with late AMD and the first generation descendants. Therefore, the authors concluded that retinal oxygen consumption was decreased in patients with early AMD and the reason was believed to be loss of oxygen consuming cellular structures in the retina. However, no significant difference of the arteriovenous difference was found between patients with late AMD and healthy individuals and it was speculated that the microcirculation had adjusted back to normal oxygen consumption. Interestingly, the arteriovenous difference in the first generation descendants was seen to be significantly higher in comparison to the controls, suggesting altered metabolism in the retina in the descendants. Although no signs of the disease were visible, it is possible that some pathological processes had already begun.

## 1.8 Vascular endothelial growth factor (VEGF)

The importance of vascular endothelial growth factor (VEGF) in retinal vasculature was recognised in the landmark paper of Michaelson (1948) in 1948. In his pioneering studies, Michaelson hypothesized that a specific factor, which he named Factor X, was responsible for abnormal retinal vessel growth and that this factor was regulated by oxygen. Decades later, Factor X was identified as VEGF (Ferrara and Henzel, 1989) and it was understood that Michaelson was observing the effects of hypoxia in retinal tissue, which causes upregulation of VEGF and consequent neovascularisation in the eye.

Many of the earlier studies on VEGF came from researchers in the field of oncology. In 1971, Folkman and colleagues isolated factors stimulating vascular growth from ascites fluid in cancer patients. This growth factor was also later identified as VEGF, a diffusible cytokine that promotes angiogenesis and vascular permeability when induced by hypoxia (Folkman et al., 1971; Folkman, 1971). In these early papers by Folkman et al., the concept of

angiogenesis inhibitors was also introduced as a possible mechanism to be utilised in cancer but likewise in neovascular retinopathies.

Further studies also confirmed the significance of VEGF in ocular neovascularisation (Adamis et al., 1994; Aiello et al., 1994; Kvanta et al., 1996). Subsequently, VEGF was identified in surgically obtained choroidal neovascularisation specimens from humans with AMD (Frank et al., 1996; Kvanta et al., 1996; Lopez et al., 1996). It has been confirmed that production of VEGF in the retina is indeed induced by hypoxia (Aiello et al., 1995; Shweiki et al., 1992) but oxidative stress has also been shown to increase the production of VEGF (Kannan et al., 2006); for more extensive review see Klettner et al. (2013).

These pivotal contributions enabled the development of the first antibody specific for VEGF that was able to inhibit the growth of tumours in a mouse model (Kim et al., 1993). In 1996, the humanization of the former mouse antibody, later named bevacizumab, was completed (Presta et al., 1997). In 2004, the bivalent recombinant humanized monoclonal antibody bevacizumab (Avastin®) and the pegylated aptamer pegaptanib (Macugen®), which both act to inhibit VEGF, received approval for use in treatment of metastatic colon cancer and neovascular AMD, respectively. In 2006, ranibizumab (Lucentis®) was also approved for intravitreal treatment of neovascular AMD and shortly after became preferred over pegaptanib due to better efficacy on visual acuity (Takeda et al., 2007). At a similar time, the unlicensed use of intravitreal bevacizumab in neovascular AMD began, because of considerably lower costs of bevacizumab. Ranibizumab is a monovalent recombinant humanized monoclonal antibody fragment, derived from the same parent antibody as bevacizumab. Ranibizumab is, therefore, much smaller in size in comparison to bevacizumab, which allows it to penetrate the retina better and conceivably cause less system side effects due to shorter half-life in systemic circulation (Ferrara et al., 2006). Two large clinical trials, the Comparison of AMD treatments trials (CATT) and the Inhibition of VEGF in age-related choroidal neovascularisation (IVAN) trial, have now evaluated the relative safety and efficacy of ranibizumab and bevacizumab. Results from the first trial, CATT, showed that both drugs had similar effects on visual acuity but bevacizumab had possibly more adverse effects, although interpretation of the safety results was uncertain (CATT Research Group, 2011, 2012). The second study, IVAN, was also not able to demonstrate that bevacizumab was inferior or non-inferior to ranibizumab (Chakravarthy et al., 2013). However, when the data from these two studies were pooled together, the non-inferiority of bevacizumab was in fact established (Chakravarthy et al., 2013). As of October 2015, bevacizumab has still not been officially approved for intravitreal use in treatment of neovascular AMD but continues to be used off-label.

Ranibizumab neutralises VEGF and its active degradation products. Clinically, this inhibition activity translates to a reduction of blood vessel

proliferation and angiogenesis induced by VEGF. Monthly intravitreally injected ranibizumab was proven highly effective in treating neovascular AMD in two large randomized, double-masked, sham-controlled clinical trials, the Minimally classic/occult trial of the anti-VEGF antibody ranibizumab in the treatment of neovascular age-related macular degeneration (MARINA) and the Anti-VEGF antibody for the treatment of predominantly classic choroidal neovascularisation in age-related macular degeneration (ANCHOR) trial. In both studies, approximately 95% of ranibizumab-treated patients with choroidal neovascularisation secondary to AMD experienced vision improvement or stabilization of visual acuity (less than 15-letter loss in visual acuity) at 12 months, in comparison with 62% of the sham-treated patients ($p<0.001$) in the MARINA study (Rosenfeld et al., 2006) and 64% of verteprofin-treated patients ($p<0.001$) in the ANCHOR study (Brown et al., 2006).

Although the therapeutic efficacy of ranibizumab was clearly demonstrated by the MARINA and ANCHOR studies, the monthly dosing regimen presented a great burden on patients and physicians. Subsequently, several studies were designed to determine if administration of ranibizumab at a less frequent interval could result in similar therapeutic gains (Busbee et al., 2013; CATT Research Group, 2011, 2012; Chakravarthy et al., 2013; Fung et al., 2007; Lalwani et al., 2009; Regillo et al., 2008; Singer et al., 2012).

The Prospective optical coherence tomography (OCT) imaging of patients with neovascular age-related macular degeneration (AMD) treated with intraocular ranibizumab (PrONTO) study was an open-label study assessing the efficacy of ranibizumab treatment with three initial monthly injections and thereafter, monthly controls with OCT-guided re-treatment schedule for a total of 24 months. Results showed improvement in visual acuity comparable to the results of the MARINA and ANCHOR studies achieved with an average of 5.6 injections over the first 12 months of the study (Fung et al., 2007) and an average of 9.9 injections over the total 24 months (Lalwani et al., 2009) but this study included only 40 participants and no control group.

The more recent Phase III, double-masked, multicenter, randomized, active treatment-controlled study of the efficacy and safety of 0.5 mg and 2.0 mg ranibizumab administered monthly or on an as-needed basis (PRN) in patients with subfoveal neovascular age-related macular degeneration (HARBOR) study involved a much larger sample size (n=1098). As the name of the study implies, the as-needed regimen of ranibizumab injections was compared to a monthly dosing regimen in order to test the non-inferiority of the as-needed regimen. After the first 12 months of the study, the patients in the as-needed group had received four less injections on average than the monthly group, or an average of 7.7 injections. However, in the comparison to the monthly regimen, the as-needed regimen failed to meet the non-inferior margin set by the HARBOR investigators (Busbee et al., 2013).

The previously mentioned CATT study, was also designed as a noninferiority trial to compare both the efficacy of monthly-dosing regimen in comparison to as-needed regimen as well as to compare ranibizumab to bevacizumab as discussed above. The preliminary results at 1 year showed no significant difference in visual acuity between the groups treated monthly or on as-needed basis, suggesting, in contrast to the HARBOR study, that the as-needed regimen was non-inferior to the monthly regimen (CATT Research Group, 2011). However, at the planned endpoint of the study at 2 years, the results showed that patients who received the treatment as needed for the entire study period or just the second year had significantly less gain in visual acuity compared with those who had followed the monthly regimen for the 2 years (CATT Research Group, 2012). The IVAN trial was also designed to compare both the efficacy of monthly-dosing regimen to as-needed regimen as well as to compare ranibizumab to bevacizumab, as previously discussed. Similar to the CATT trial, the IVAN trial showed a slightly better outcome in the monthly treated group in comparison to the as-needed group, although inferiority could not be established for the as-needed regimen (Chakravarthy et al., 2013). However, when the data from the CATT and IVAN studies were pooled together, the as-needed regimen was inferior to the monthly regimen (Chakravarthy et al., 2013).

The most recently approved intravitreal treatment for neovascular AMD, aflibercept (Eylea®), has shown very promising results in clinical trials (Heier et al., 2012). Aflibercept is a soluble decoy receptor fusion protein that has been engineered to have much higher binding affinity to VEGF compared to both ranibizumab and bevacizumab and much longer half-life (Holash et al., 2002), making it possible to administer the drug bimonthly after the first three monthly injections, with comparable effects on visual acuity as monthly treatments of ranibizumab (Heier et al., 2012). It has also been shown to have similar safety and tolerability to ranibizumab (Heier et al., 2012), which makes aflibercept advantageous in terms of the potential of cost savings and decreased frequency of use with the lower treatment burden and lower cumulative risk of injection-related adverse effects.

In March 2007, ranibizumab became a treatment option at the Department of Ophthalmology at the Landspitali University hospital in Iceland for patients with neovascular AMD. All patients were treated with initial three monthly injections and thereafter according to as-needed regimen (described further in chapter 3.2.2). The centralised eye service in Iceland as well as the confined population of approximately 320 000 makes it possible to estimate the service load and costs on a nationwide scale involved in offering anti-VEGF drugs to patients with neovascular AMD.

For further information on treatment for neovascular AMD see extensive reviews by Lally et al. (2012) and Frampton (2013).

## 1.9 Epidemiology of AMD

Worldwide, AMD is the third most common cause of blindness, after cataract and glaucoma (Resnikoff et al., 2004). In the industrialised countries, AMD is, however, the most common cause of severe visual impairment and irreversible blindness among people 50 years of age or older (Congdon et al., 2004; Gunnlaugsdottir et al., 2008; Hong et al., 2013; Klein et al., 2013b).

### 1.9.1 Prevalence of AMD

The prevalence of AMD has been studied extensively in the past decades in several large population-based epidemiological studies (Jonasson et al., 2003, 2011; Klein et al., 1992; Krishnan et al., 2010; Mitchell et al., 1995; Spanish Eyes Epidemiological (SEE) Study Group, 2011; Vingerling et al., 1995b). A meta-analysis of population-based studies from three continents including persons aged 40 years and older of Caucasian origin (Smith et al., 2001) estimated prevalence of early AMD to be 6.8% and late AMD 1.5%. In the Reykjavik Eye Study, the prevalence in persons aged 50 years and older was 17.9% for early AMD and 3.5% for late AMD (Jonasson et al., 2003). More recently, the Age, Gene/Environment Susceptibility Reykjavik Study showed the prevalence of early AMD to be 21.3% and late AMD 5.7% in an older cohort, but all participants were 66 years of age and older (Jonasson et al., 2011).

As expected, prevalence of all stages of AMD increases with age (Jonasson et al., 2003, 2011; Smith et al., 2001), and after 75 years of age there is an exponential increase (Friedman et al., 2004).

Neovascular AMD has generally been found to be more prevalent than geographic atrophy, with data ranging from approximately 55-75% of all late AMD cases depending on population (Jonasson et al., 2011; Klein et al., 1992; Mitchell et al., 1995; Vingerling et al., 1995b). In the meta-analysis from three continents, the prevalence of neovascular AMD in the oldest age-group, participants aged 85 years and older, was 8.8% (n=521) (Smith et al., 2001) but it was 11.4% (n=436) in the same age-group in Iceland (Jonasson et al., 2011). These high prevalence rates in Iceland are among the highest reported in the world; only the Inuits in Greenland are reported to have higher prevalence of late AMD (Andersen et al., 2008).

### 1.9.2 Incidence of AMD

The incidence of AMD has also been estimated in large population-based studies (Jonasson et al., 2005; Klein et al., 2007; van Leeuwen et al., 2003; Wang et al., 2007). Comparably to the prevalence, the incidence of AMD, and more specifically that of neovascular AMD, increases with age (Jonasson et al., 2005; Klein et al., 2007; van Leeuwen et al., 2003; Wang et al., 2007). The cumulative incidence of neovascular AMD in the Beaver Dam Eye Study with the study population aged 43 years and older at baseline was at 5-years 0.4% (Klein et al.,

1997), at 10-years 0.9% (Klein et al., 2002) and at 15-years 2.0% (Klein et al., 2007). The mean annual incidence of neovascular AMD for the same periods was 0.08%, 0.09% and 0.13%, respectively. The mean annual incidence of neovascular AMD in the Blue Mountains Eye Study was 0.10% for the whole study population 49 years and older at baseline (calculated from 5-year cumulative incidence rates) (Mitchell et al., 2002). In the Reykjavik Eye Study, the mean annual incidence of neovascular AMD was only 0.03% (calculated from 5-year cumulative incidence rates), but the low incidence rates were in part explained by the low number of participants (Jonasson et al., 2005).

### 1.9.3 Economic impact of AMD

Visual acuity is usually insignificantly affected in early AMD. However, despite normal visual acuity, many patients can experience loss in contrast sensitivity (Kleiner et al., 1988; Stangos et al., 1995), decreased rate of recovery after photostress (Binns and Margrain, 2007; Sandberg and Gaudio, 1995) and reduced dark adaptation (Dimitrov et al., 2012). When patients are diagnosed with late AMD, either geographic atrophy (chapter 1.5.3.1) or neovascular AMD (chapter 1.5.3.2), they normally have some noticeable vision loss, with the central vision predominantly affected (Coleman et al., 2008; Owen et al., 2003). With the anti-VEGF treatments for neovascular AMD, which have been available since 2006 (reviewed in chapter 1.8), the vision loss is in many cases reversible to some extent and the incidence of severe visual impairment due to AMD is decreasing. As an example, in persons 50 years and older in Denmark, the annual incidence rate of legal blindness attributable to AMD decreased from 52.2 cases per 100 000 in the year 2000 to 25.7 cases per 100 000 in 2010, equivalent to a significant reduction of 50%, where the greatest part of this reduction occurred after the year 2006 (Bloch et al., 2012). Additionally, the conclusion of the AREDS study in 2001, that dietary supplements can reduce the risk of AMD progression (Age-Related Eye Disease Study Research Group, 2001), has now led to widespread use of specific non-prescription formulation of vitamin C and E, beta carotene, zinc and copper in persons with AMD.

According to population projections, a substantial enlargement of the older age-groups is anticipated in the next decades. Consistent with the aforementioned population-based studies, this would be expected to cause a substantial increase in the prevalence of AMD as well as the morbidity from the disease (Finger et al., 2011; Minassian et al., 2011; Rein et al., 2009). However, predictions remain extremely challenging as the exact nature of the disease and its risk factors is not yet known and, as discussed above, the morbidity from the disease appears to be decreasing due to the use of anti-VEGF treatments. To complicate projections even more, some data have shown a lower incidence of early AMD with later birth and period cohorts (Klein R. et al., 2008) compared with the larger population-based studies

previously described. This trend is possibly related to more awareness of the disease and lower exposure to known and perhaps unknown risk factors. Currently, in persons 55 years and older, the five-year risks of progressing to late AMD are estimated to range from a 0.5% in those who have normal aging changes in the fundus to approximately 50% risk for those who have intermediate AMD as well as other known risk factors (Ferris et al., 2013).

The economic impact of AMD is already substantial, not solely due to the marked morbidity from the disease itself, but also as the available treatment for neovascular AMD increases ophthalmology service load and drug costs greatly (Keenan et al., 2012). Moreover, the frequent follow-up visits and repeated intravitreal injections for patients could be expected to decrease their quality of life. However, studies have shown that the benefit of treatment outweighs the burden of frequent visits and patients generally report improvement in their quality of life, more specifically the vision-specific quality of life (Solomon et al., 2014).

# 2 Aims

The general aims of this thesis are to test whether retinal oxygen metabolism in neovascular AMD is different from a healthy cohort and determine the economic burden of neovascular AMD in Iceland. The more specific aims are:

1. *Methodology*:
    a. Standardize data acquisition and test reliability of a new retinal oximeter (**Paper II**).
    b. Establish a normative database for retinal oximetry values in a healthy cohort over a long age span (**Paper I**).
2. *Pathophysiology*: Determine retinal vessel oxygen saturation in neovascular AMD and compare with healthy cohort (**Paper III**).
3. *Epidemiology*: Assess the population-based annual incidence of neovascular AMD and estimate the treatment load of intravitreal ranibizumab injections in a defined population of persons 60 years and older (**Paper IV**).

# 3 Materials and methods

## 3.1 The Retinal Oximetry Study (Papers I-III)

Appropriate ethical approvals for the study were obtained from the National Bioethics Committee of Iceland (06-084 with later amendments) and the Icelandic Data Protection Authority (2006-070338 with later amendments) following the principles of the Declaration of Helsinki. All participants signed informed consent before enrollment in the study.

### 3.1.1 Study populations

#### *3.1.1.1 Healthy persons (Papers I and II)*

For the study on the normal population, allegedly healthy Caucasian persons 18 years and older were recruited. A total of 148 individuals were examined but 28 had to be excluded according to the exclusion criteria in **Table 1**. The remaining 120 persons were included in the study. In Paper I all the 120 individuals were analysed but in Paper II only a subset of the participants aged 18-30 years old were analysed, a total of 26 persons.

**Table 1**. Exclusion criteria for participants in the study on retinal oximetry in a healthy population and the number of subjects excluded due to each criterium.

| | |
|---|---|
| Eye | Retinal diseases (n=12)<br>Optic nerve diseases (n=6)<br>Any eye disease that could affect the quality of images[a] (n=1)<br>Previous history of eye trauma (n=0)<br>Known or suspected adverse effect of pupil dilation (n=0) |
| System diseases | Diabetes mellitus (n=2)<br>Severe cardiovascular diseases (n=0)<br>Severe respiratory diseases (n=0) |
| Maternity | Pregnant (n=0)<br>Breastfeeding (n=0) |
| Images | Low image quality for both eyes (n=5)<br>Missing data from both eyes (n=2) |

[a]Participants with intraocular lenses after cataract surgeries were not excluded from the study.

Distribution of age and gender of the 120 persons aged 18-80 years can be seen in **Table 2**. The 26 persons used in the subgroup analysis (Paper II) were from the youngest age-group in, and two of the youngest subjects from the next age-group were included as well.

**Table 2.** Total number of healthy Caucasian participants in each age group in the study, subdivided by gender. Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, Association for Research in Vision and Ophthalmology).

| Age (years) | Males | Females | Total |
|---|---|---|---|
| 18-29 | 4 | 20 | 24 |
| 30-39 | 12 | 12 | 24 |
| 40-49 | 7 | 13 | 20 |
| 50-59 | 5 | 15 | 20 |
| 60-69 | 10 | 13 | 23 |
| 70-80 | 6 | 3 | 9 |
| Total | 44 | 76 | 120 |

In Paper I, 225 oximetry images from 225 eyes of 120 participants were analysed but both eyes of a participant were needed and included solely for the comparison between right and left eye (n=105). For all other analyses, only one eye for each subject was randomly selected. In those cases where a person had only one eligible eye in the study, that eye was entered for the analyses. The median age of the 120 participants was 47 years (males 49 years; females 44 years). Four females (20, 24, 72, 76 years) could not be included in the multivariate analyses as data were missing on blood pressure, intraocular pressure or smoking status.

In Paper II, 104 oximetry images from 26 eyes of 26 participants were analysed, see further in chapter 3.1.4. For evaluation of repeatability and effect of vessel location within image, all 26 participants could be used for analyses. For determination of topographical variance between retinal quadrants, only participants who had a measureable arteriole or venule in each quadrant could be included in the analysis. Therefore, data from 21 subjects were used for the evaluation of arteriolar oxygen saturation in different quadrants and data from 19 subjects for venular oxygen saturation.

#### *3.1.1.2 Neovascular AMD patients (Paper III)*

For the study on retinal oximetry in neovascular AMD (Paper III), persons with treatment-naïve neovascular AMD in at least one eye were recruited. Criteria for the diagnosis of neovascular AMD included any signs of choroidal neovascularisation located under the retinal pigment epithelium, or under or within the retina as well as serous, drusenoid, fibrovascular or hemorrhagic pigment epithelial detachment, but patients with definite disciform changes were excluded as well as those who had any of the exclusion criteria relevant to the healthy population (see **Table 1**, chapter 3.1.1). Differentiation between the various subtypes of choroidal neovascularisation was not performed. As with the healthy population, all AMD subjects were Caucasian.

This study compared 46 neovascular AMD patients aged 66-95 years and 120 healthy individuals aged 18-80 years (thereof 12 individuals 66 years or older). Four AMD patients and three healthy subjects could not be included in the multivariate analyses due to missing data on blood pressure values or current smoking status. See further information on age, gender distribution and number with high blood pressure (systolic blood pressure above 140 mmHg and/or diastolic blood pressure above 90 mmHg) in **Table 3**.

**Table 3.** Demographic description of subjects in the comparative study on neovascular AMD patients and healthy persons.

| | **Neovascular AMD patients (n=46)** | **Healthy persons (n=120)** |
|---|---|---|
| Age (years) | 79±7 (mean±SD)<br>80 (median)<br>66-95 (range) | 45±17 (mean±SD)<br>47 (median)<br>18-80 (range) |
| Gender (males:females) | 10:36 | 44:76 |
| Number with high blood pressure | 27 | 19 |

In this study on neovascular AMD (Paper III), only one image of each eye from every participant was analysed. For the healthy persons, randomization was used to select one eye for the analysis of the oximetry images (see further chapter 3.1.1.1). For the AMD patients, an eye with treatment-naïve neovascular AMD was used for the analysis. However, if the patient had treatment-naïve neovascular AMD in both eyes, only one eye was randomly chosen for each subject.

### 3.1.2 Study protocol (Papers I-III)

All participants went through a standard study protocol. Information on self-reported medical history, smoking history and current medications was gathered from a questionnaire filled out with each subject. The examination included measurements of blood pressure and heart rate (Omron M6 Comfort (HEM-7000-E), Omron Healthcare Europe, Hoofddorp, the Netherlands), finger pulse oximetry (Ohmeda Biox 3700, Ohmeda, Boulder, CO, USA), best corrected visual acuity (Snellen chart) and intraocular pressure (IOP; iCare TAO1 Tonometer, Tiolat Oy, Helsinki, Finland). Ocular perfusion pressure (OPP) was calculated according to Equation 1 (see chapter 1.2).

Dilation of pupils was achieved with 1% tropicamide (Mydriacyl; S.A. Alcon-Couvreur N.V., Puurs, Belgium) and for some subjects supplemented with 10% phenylephrine hydrochloride (AK-Dilate; Akorn Inc., Lake Forest, Illinois, USA). The anterior segment of the eye was examined before pupil dilation. After dilation of the pupil, a standard 50° colour fundus image (Zeiss FF 450*plus* fundus camera, Carl Zeiss Meditec AG, Jena, Germany) was

taken of all healthy subjects as well as most AMD patients. Finally, the participants underwent oximetry.

### 3.1.3 Retinal oximeter

The Oxymap T1 retinal oximeter (Oxymap ehf., Reykjavik, Iceland) is a dual-wavelength non-invasive spectrophotometer. It is composed of two digital cameras (Insight IN1800, 1600 x 1200 square pixels, Diagnostic Instruments Inc., MI, USA), an image splitter, two narrow band-pass filters and a custom-made optical adapter, which couples the oximeter to a fundus camera (Topcon TRC-50DX, Topcon Corporation, Tokyo, Japan) (**Figure 13)**.

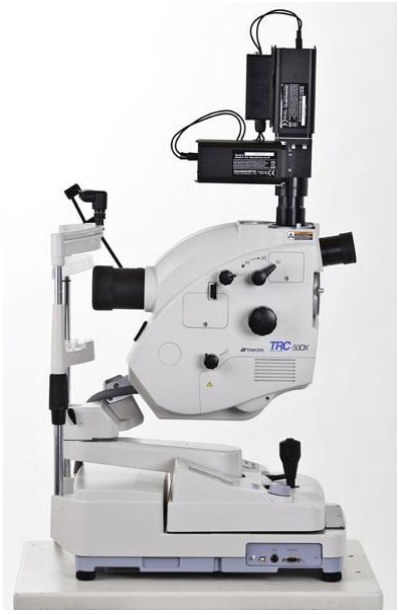

**Figure 13.** The retinal oximeter Oxymap T1 (Oxymap ehf., Reykjavik, Iceland). Reprinted from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

The two narrow 5 nm bandpass filters (full width at half maximum transmittance) allow the oximeter to simultaneously acquire two monochrome fundus images of the same area of the retina at two different wavelengths of light, 570 and 600 nm (**Figure 14**). At the isosbestic wavelength at 570 nm (**Figure 14a**), the arterioles and venules are similarly dark, whereas at the non-isosbestic wavelength at 600 nm (**Figure 14b**), the light absorbance decreases with increased oxygen saturation and arterioles appear much brighter than venules. The oximeter also has a broader 80 nm bandpass filter in the light path of the fundus camera (585 nm center wavelength), which only allows light between 545 nm and 625 nm to exit the camera lens and thereby limits unnecessary light exposure to the subjects' eyes.

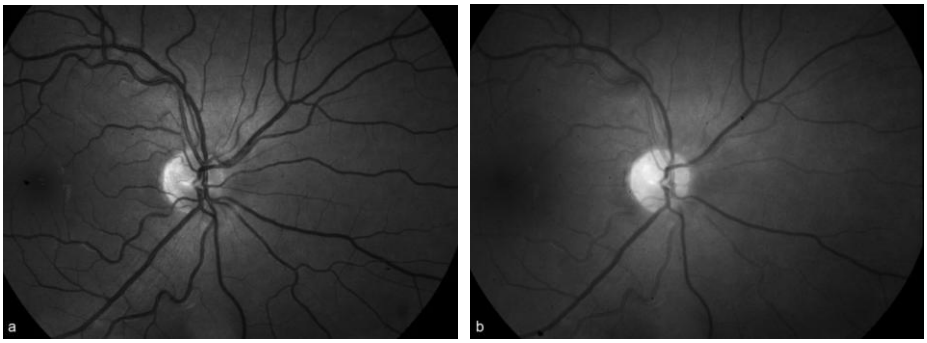

**Figure 14**. Two monochrome images simultaneously acquired by the Oxymap T1 retinal oximeter. (a) Image taken at 570 nm, which is the reference isosbestic wavelength and therefore, insensitive to oxygen saturation. (b) Image taken at 600 nm, which is the oxygen sensitive wavelength. It can be seen in figure (b) how the light absorbance of the vessels changes with different oxygen saturation; i.e. there is a clear difference between of arterioles and venules. Reprinted from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

### 3.1.4 Oximetry imaging

For oximetry imaging, the participant was at first seated comfortably in front of the fundus camera and registered in the database. The room was darkened, with the only light sources from the fundus camera and the computer screen. The same experienced research scientist took all the oximetry images, first of the right eye and then of the left eye. The fundus was illuminated with the lowest possible setting of the aiming light, which allowed appropriate alignment of the fundus camera. The small aperture setting was turned on, and the small pupil setting was used in selected cases if needed (registered by the photographer). The flash intensity was set to 50 Ws (Watt seconds). Generally, five images were taken of each eye under different alignment according to the protocol seen in **Figure 15** but occasionally further images were added to acquire an image of sufficient quality for every area. The time between images of the same eye, and thereby flashes, was on average about 30 seconds.

In Papers I and III, image 2 (or 5) in **Figure 15** was selected and analysed, whereas in Paper II images 2-5 in **Figure 15** were analysed.

### 3.1.5 Image processing

The oximetry images were processed with a specialised software (Oxymap Analyser software 2.2.1, version 3847, Oxymap ehf., Reykjavik, Iceland). With the correct calibration of the oximeter, the software can automatically process the two monochrome images and presents the results as a pseudocolour fundus map. The calibration of constants *a* and *b* (from Equation 7 in chapter 1.3.2) for Oxymap T1 was accomplished by using the Oxymap T1 to measure the optical density ratios in healthy subjects and match the results with

saturation measurements from a study by Schweitzer et al. (1999). Schweitzer et al. used calibrated imaging spectroscopy to measure oxygen saturation in whole blood *in vivo* and *in vitro*. They found mean oxygen saturation to be 92.2% in retinal arterioles and 57.9% in venules. By matching the optical density ratios measured with Oxymap T1 with the oxygen saturation results from Schweitzer et al., the calibration constants for Equation 7 were found to be $a$=-1.1755 and $b$=1.1917.

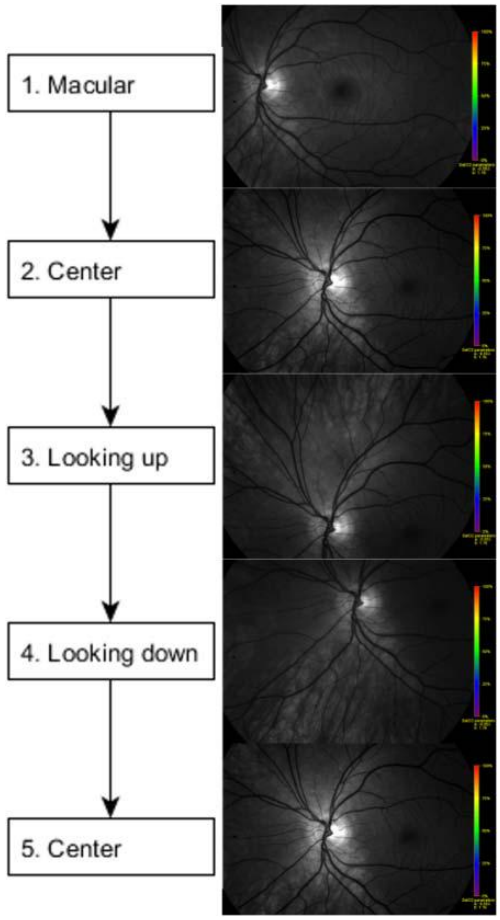


**Figure 15.** Protocol for fundus image acquisition. First, a fundus image centered on the macula (1), then an image centered on the optic disc (2), followed by images where the subject looked up (3), then down (4) and finally a repetition of image 2 with the optic nerve head in the center (5). Reprinted from Paper II (Invest Ophthalmol Vis Sci, 53(4), 1729-1733, ©2012, with permission of Association for Research in Vision and Ophthalmology).

**Figure 16** shows an example of a pseudocolour map from the right eye of one of the study participants. The colours in the image, lining the retinal vessels, indicate the oxygen saturation of each particular vessel according to the scale on the right side of the figure. Arterioles are normally orange to red, indicating

oxygen saturation of approximately 90-100%. The colour of the venules can vary from blue to yellow but is generally green, indicating oxygen saturation approximately 50-60%.

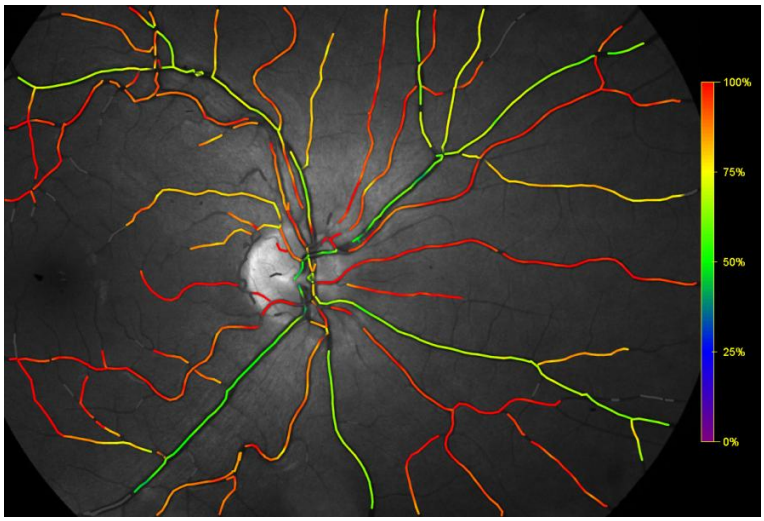


**Figure 16.** Pseudocolour fundus map automatically generated by the Oxymap T1 oximeter and the Oxymap Analyser software. Colours indicate oxygen saturation in retinal vessels according to the scale to the right of the image. Reprinted from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

The oximeter can also measure the diameter of the vessel. With increased diameter of both retinal arterioles and venules, studies have shown an artifactual decrease in measured oxygen saturation values (Beach et al., 1999; Gottfredsdottir et al., 2011; Hammer et al., 2008). Within each eye a considerable range of diameters was found for both arterioles and, in particular, for venules, where diameters from 8 pixels (determined as the narrowest vessel diameter included) up to 25 pixels were seen. One pixel in vessel diameter has been shown to be equivalent to approximately 9.3 micrometers (Blondal et al., 2011). Within this large range there is a certain effect of vessel diameter on saturation calculations, which has to be accounted for. Therefore, a correction of saturation measurements for vessel width was performed on all vessels.

The correction factor was determined in a separate analysis of 21 eyes from 21 healthy individuals 18-40 years old using the same version of the oximetry software. The main superotemporal arteriole and venule in each eye were analysed by selecting 20-50 pixels long vessel segments, 8 pixels or larger in diameter, on both sides of the bifurcation of the vessels from the primary branch (*pri*) and the secondary branches (*sec*). Precisely at the top of the bifurcation, an exclusion mark of 15 pixels in diameter was placed so that the branching itself would not create spurious results. Oxygen saturation

($SaO_2$) was assumed to be the same on both sides of the bifurcation. This assumption was made for both arterioles and venules, even though it might not be completely correct for venules. Consequently, the mean oxygen saturation of the secondary branches ($sec_1$, $sec_2$) was assumed to be equal to the oxygen saturation of the primary branch (*pri*) according to Equation 8:

$$SaO_{2(cor,pri)} = \frac{SaO_{2(cor,sec1)}+SaO_{2(cor,sec2)}}{2}, \qquad \textit{Equation 8}$$

where $SaO_{2(cor)}$ is the oxygen saturation for primary (*pri*) and secondary (*sec*) branches after correction for vessel diameter. For correction of vessel diameter Equation 9 was used:

$$SaO_{2(cor)} = k * \left(d - \bar{d}\right) + SO_{2(uncor)}, \qquad \textit{Equation 9}$$

where *k* is the correction factor, $d$ is the vessel diameter, $\bar{d}$ is the mean vessel diameter for the primary vessels and $SaO_{2(uncor)}$ is the measured oxygen saturation without correction for vessel diameter. The mean diameter for arterioles was 11.24 pixels and for venules 14.46 pixels. Equations 8 and 9 were then combined to solve for the correction factor *k* as follows:

$$k = \frac{SaO_{2(uncor,sec1)}+SaO_{2(uncor,sec2)}-2*SaO_{2(uncor,pri)}}{2*d_{pri}-d_{sec1}-d_{sec2}}, \qquad \textit{Equation 10}$$

By using Equation 10**,** the correction factor *k* could be calculated for each of the 21 eyes. This resulted in a median of 1.17% per pixel for arterioles and 1.15% per pixel for venules, which was averaged to 1.16% per pixel for both arterioles and venules to simplify future calculations. This was considered acceptable since the original results were within the standard deviation of each other. As a result, all oxygen saturation measurements in both arterioles and venules were corrected by adding 1.16% to the saturation value for each pixel by which the vessel was wider than the fixed mean diameter values (11.24 pixels for arterioles and 14.46 pixels for venules). Similarly, 1.16% was subtracted for each pixel by which the vessel was narrower than the mean diameter.

In summary, the original oxygen saturation values from the oximetry software were recalculated for every vessel of each analysed image, to incorporate the new calibration constants as well as the vessel diameter correction factor.

### 3.1.6 Image analysis

For each subject in Papers I and III, the first good quality image with the optic disc in the center was used for analysis. In Paper II, images of other alignments were analysed as well under specific settings as described later in this chapter. Measurements of oxygen saturation were performed in all major retinal arterioles and venules 8 pixels or larger in diameter. As vessels to be

analysed had to be selected manually by the user, a detailed protocol was made for standardisation of vessel selection based on their width, length and location within the image (**Table 4**). Consequently, only a specific segment of each vessel was measured. **Figure 17** shows how the software puts a white overlay on the selected vessel segment, whereas all excluded vessel segments remain coloured according to their saturation and all vessels smaller than 8 pixels have a gray colour.

**Table 4.** Criteria for choosing retinal vessel segments for measurement of oxygen saturation with the Oxymap T1 retinal oximeter. Vessel width in pixels (1pixel≈9.3µm (Blondal et al., 2011)), vessel length in pixels (count) and location of chosen vessel segments within the pseudocolour fundus image were selected in a standardised manner. Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

| | |
|---|---|
| Retinal vessel width | 8 pixels or wider |
| Retinal vessel length | 50-200 pixels (preferably as close to 200 pixels as possible) |
| Start vessel selection | As close to the optic disc as possible but always exclude at least 15 pixels next to the optic disc (or a bright area surrounding it) |
| End vessel selection | After length of 200 pixels but never closer than 30 pixels to the edge of the image<br>If vessel branches ≥50 pixels from the selection start |
| If vessel branching occurs <50 pixels from the selection start and if the smaller of the daughter vessels is:<br>a) 6 pixels or less in diameter<br>b) wider than 6 pixels | a) Ignore the vessel branching and analyze the segment from the optic disc<br>b) Start the analysis from the vessel branching and measure segments further away from the optic disc |
| Exclude vessel segments specifically | If the background points are affected by extremes in brightness (undetected nearby vessels, laser scars, hemorrhages etc.) |

After the manual selection of vessel segments to be analysed, the Oxymap Analyser software automatically measured the oxygen saturation within each vessel selected, one at a time. In Papers I and III, the mean and standard deviations of oxygen saturation measurements of the entire fundus were determined. In Paper II, analyses were performed on particular vessels (repeatability of measurements), retinal quadrants (topographical variance) or superior and inferior parts of the fundus (vessel location in image).

#### *3.1.6.1 Repeatability of measurements*

For determination of repeatability of the oxygen saturation measurements for the Oxymap T1 retinal oximeter, two separate images of the same eye (images 2 and 5 in **Figure 15**) were analysed in a standardised manner. The repeatability of oxygen saturation measurements was determined for the average values for all arterioles and venules in an eye as well as for a single vessel segment from each subject. For the single vessels, a large vessel in the superotemporal quadrant was chosen.

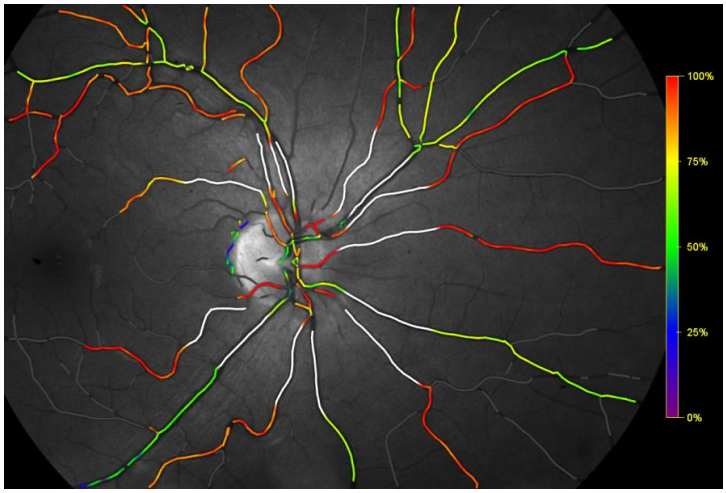


**Figure 17.** Pseudocolour fundus map with manually selected vessel segments intended for analysis. The Oxymap Analyser software is programmed to put a white overlay on vessel segments manually selected to be analyzed. This image is for demonstration purposes and in practice, each vessel segment (50-200 pixels in length) was selected in a standardised manner according to the protocol and analysed one at a time. The oximeter was set to detect all vessels 8.0 pixels or more in diameter, smaller vessels appear gray in figure. Reprinted from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

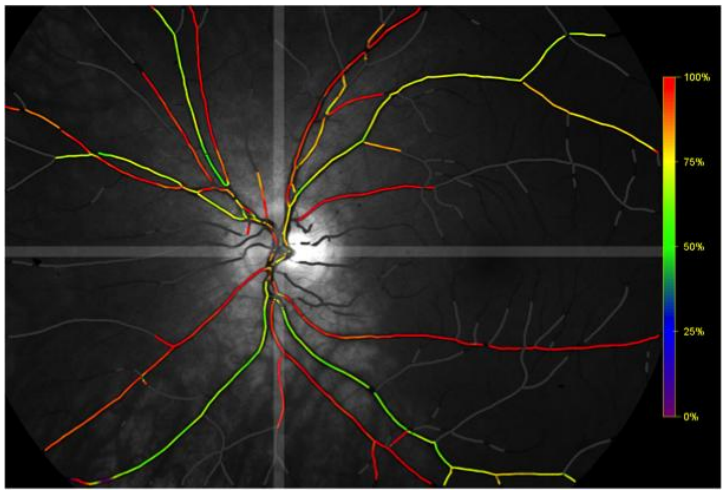


**Figure 18**. Pseudocolour fundus map showing how the retinal quadrants were determined. Reprinted from Paper II (Invest Ophthalmol Vis Sci, 53(4), 1729-1733, ©2012, with permission of Association for Research in Vision and Ophthalmology).

### *3.1.6.2 Topographical variance*

For evaluation of topographical variance of oxygen saturation, images with the optic disc in the center (image 2 in **Figure 15**) were analysed after classifying all vessels into respective retinal quadrants according to **Figure 18**. This

analysis was carried out for the entire group of healthy subjects (Paper I) as well as the subgroup of the youngest participants (Paper II).

### *3.1.6.3 Vessel location in image*

To estimate if oxygen saturation measurements were affected by the location of the vessel within an image, the same vessel segments were analysed on two different images from the same eye. Vessels in the superior part of the image (belonging to the two superior quadrants on **Figure 18**) were measured in an image with the optic disc in the center (image 2 in **Figure 15**) and in an image where the optic disc and the superior vessel arcades had moved down in the image as the subject gazed up (images 3 in **Figure 15**). See the effect of upgaze on oximetry imaging in **Figure 19**.

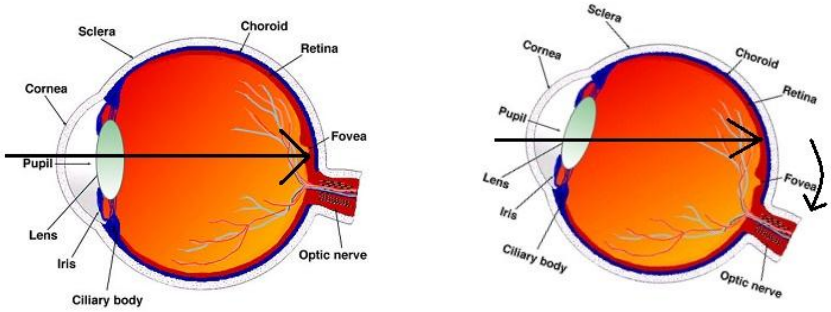


**Figure 19**. The effect of upgaze for retinal imaging. The optic nerve and the superior vessel arcades move downwards in the image, when the patient looks upwards. The horizontal arrow represents the light from the oximeter striking the retina. The arrow directed downwards shows the optic disc moving downwards in an image taken during upwards gaze. ©2015, http://webvision.med.utah.edu/. Modification (arrows and rotation) with permission from Webvision.

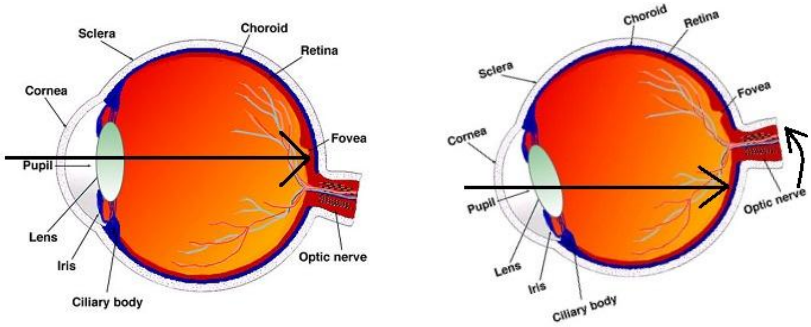


**Figure 20**. The effect of downgaze for retinal imaging. The optic nerve and the superior vessel arcades move upwards in the image, when the patient looks downwards. The horizontal arrow represents the light from the oximeter striking the retina. The arrow directed upwards shows the optic disc moving upwards in an image taken during downwards gaze. ©2015, http://webvision.med.utah.edu/. Modification (arrows and rotation) with permission from Webvision.

Conversely, vessels in the inferior part of the image (belonging to the two inferior quadrants on **Figure 18**) were also measured in an image with the optic disc in the center (image 2 in **Figure 15**) as well as in an image where the optic disc and the superior vessel arcades had moved up as the subject gazed down (image 4 in **Figure 15**). See the effect of downgaze on oximetry imaging in **Figure 20**.

### 3.1.7 Statistical analyses

All statistical analyses for Papers I, II and III were performed using Prism, version 5.0 (GraphPad Software Inc., La Jolla, California, USA) and for Papers I and III, the R software package, version 2.14.1 (The R Foundation for Statistical Computing, www.r-project.org) was also used. For all analyses, $p<0.05$ was considered statistically significant. Several different types of statistical tests were used in the various analyses. The choice of test used for each analysis is stated together with the results in chapter 4. However, the multivariate analyses in Papers I and III, the one-way analysis of variance (ANOVA) in Paper II as well as the simple linear regression model in Paper III require a more detailed description, which will be addressed here.

In Paper I, the multivariate analyses for arterioles, venules and arteriovenous difference included age, gender, current smoking status, finger pulse oxygen saturation (measured) and ocular perfusion pressure (calculated according to Equation 1 in chapter 1.2). As ocular perfusion pressure is derived from systolic, diastolic and intraocular pressure measurements, those three variables cannot be added individually into the multiple linear regression models together with ocular perfusion pressure, which was considered the most relevant variable of the pressure variables. The interaction between age and gender was also included in the models for arterioles and venules but the complete multiple linear regression model for retinal vessel oxygen saturation ($SaO_2$) can be seen in Equation 11:

$$SaO_2 = x_0 + x_1 * A + x_2 * G + x_3 * SS + x_4 * SpO_2 + x_5 * OPP + x_6 * A * G, \quad Equation\ 11$$

where the $x_i$ are the coefficients in **Table 12**, **Table 13** and **Table 14**, $A$ is age in years, $G$ is gender (0=male, 1=female), $SS$ is smoking status (0=non-smoker, 1=current smoker), $SpO_2$ is the finger pulse oximeter reading in percentage and $OPP$ is the ocular perfusion pressure in mmHg. The difference between males and females is represented by the coding of the gender variables (0=male, 1=female) and the coefficient for gender ($x_2$) as well as the interaction of age and gender ($x_6$), where relevant.

In Paper II, one-way ANOVA was used to determine the repeatability of oxygen saturation measurements. ANOVA was used to partition the total variance into variance between individuals and variance between repeated measurements of the same individual. Taking the square root of the latter

gives an estimation of the standard deviation of repeated measurements of the same individual.

In Paper III, a simple linear regression model was used to test if age had any effect on the retinal vessel oxygen saturation in arterioles, venules and arteriovenous difference. Separate models were made for the AMD patients and the healthy controls and analysis of covariance (ANCOVA) used to test the difference between the age relationships for the two groups (difference in slopes).

Moreover, in Paper III, a multiple linear regression analysis was performed for the AMD patients and the healthy persons of the control group to correct for the effect of age difference between the study groups, as well as adjust for other possible confounding factors. The variables included in the models for arterioles, venules and the arteriovenous difference were as following: study group, age, gender, systolic blood pressure, diastolic blood pressure, heart rate, finger pulse oximetry, current smoking status and diagnosis of hypertension. The interaction between age and gender as well as the interaction between age and study group was also included in the model. The original model for retinal vessel oxygen saturation ($SaO_2$) was expressed as Equation 12:

$$SaO_2 = x_0 + x_1 * SG + x_2 * A + x_3 * G + x_4 * SP + x_5 * DP + x_6 * HR + x_7 * SpO_2$$
$$+x_8 * SS + x_9 * HTN + x_{10} * A * G + x_{11} * A * SG, \qquad \textit{Equation 12}$$

where the $x_i$ *(or* $y_i$ *or* $z_i$*)* are the coefficients in **Table 15**, *SG* is the study group (1=AMD, 0=healthy), *A* is age in years, *G* is gender (0=male, 1=female), *SP* is systolic blood pressure in mmHg, *DP* is diastolic blood pressure in mmHg, *HR* is heart rate in beats per minute, $SpO_2$ is the finger pulse oximeter reading in percentage, *SS* is smoking status (1=current smoker, 0=non-smoker), *HTN* is selfreported diagnosis of hypertension (1=with hypertension, 0=not diagnosed with hypertension). The difference between AMD patients and healthy controls is represented with the coding of the study group variables (1=AMD, 0=healthy), the coefficients for study group ($x_1$) and the interaction of age and study group ($x_{11}$). For determination of variables into the final models, a backward as well as forward selection of the variables was performed and these analyses led to same results. The final models for arterioles, venules and arteriovenous difference with the included variables and the relevant coefficients are shown in **Table 15**.

## 3.2 The Epidemiological Study on AMD (Paper IV)

The study adhered to the tenets of the Helsinki Declaration and appropriate ethical approvals were obtained from the National Bioethics Committee of Iceland (98-015 with later amendments), Icelandic Data Protection Commission (98-030122 with later amendments) and the Medical Ethics Board of Landspitali – the National University Hospital of Iceland (74/97-98).

### 3.2.1 Study population

Paper IV describes a prospective study that was conducted on patients with treatment-naïve neovascular AMD, who started treatment with intravitreal injections of ranibizumab during the time period from March 2007 throughout December 2009. In total, 439 participants were included in the study according to the inclusion criteria in **Table 5**.

**Table 5.** Inclusion criteria for participants in the study on population-based incidence of neovascular AMD.

| | |
|---|---|
| 1. Consent | *Patients required to sign informed consent* |
| 2. Age | *≥60 years* |
| 3. Disease status | *a. Neovascular AMD*[a]<br>*b. Signs of presumed recent disease progression as defined by (≥1 or more items):*<br>*(i) Recent loss of vision*<br>*(ii) New hemorrhage*<br>*(iii) Signs of intra-/subretinal fluid on optical coherence tomography (OCT)*<br>*(iv) Increase in lesion size on fluorescein angiography* |

[a]The diagnosis of neovascular AMD was based on fundus biomicroscopy, OCT and in selected cases, fluorescein angiography.

In 78 patients (18%), the second eye progressed to neovascular AMD during the study period and was thereby entitled to ranibizumab treatment. In those cases, both eyes were included in the study but handled separately for all follow-up visits and retreatment decisions. The median interval between the start of treatment in each of the two eyes was 3 months. Right eyes were 246 (48%). In total, 517 eyes from 439 patients (164 males and 275 females) were enrolled in the study. Mean age of patients at baseline was 79±7 years (males 79±7 and females 80±7; median 80 years, range 61-97 years).

All the participants consented to initiate treatment with a loading dose of three monthly intravitreal injections of 0.5 mg ranibizumab. Nine patients (9 eyes) did not complete this initial treatment; 5 patients decided to discontinue treatment after the first injection and 4 patients died during the first three months of the study period. All of these 9 patients were included in the assessment of the incidence but excluded from the analysis of number of injections.

### 3.2.2 Study details

Baseline examination included ophthalmologist's assessment of fundus biomicroscopy, optical coherence tomography (OCT) scans generated by the macular thickness map protocol of the Stratus OCT (Carl Zeiss Meditec, International, Germany) and in selected cases fluorescein angiography (Zeiss FF 450*plus* fundus camera, Carl Zeiss Meditec AG, Jena, Germany; intravenous 5 millilitres of 10% sodium fluorescein, (Anatera®, Alcon, Denmark)). Best corrected visual acuity (BCVA) on Snellen chart was assessed

at baseline and at all follow-up visits. After the baseline examination and confirmation of neovascular AMD, participants received their first intravitreal treatment, usually on the same day or the following day, but always within a week. All injections were performed in operating rooms following general guidelines for surgical procedures. Four weeks after the initial three-month loading dose scheme, the patients were scheduled for the first follow-up examination and possible retreatment as determined by the consultant ophthalmologist after fundus biomicroscopy and OCT scans. A set of criteria was used as a guide to the retreatment decision-making (**Table 6**), but these were not prescriptive and the eventual decision to retreat was based on individual consultant's judgment. If clinical evidence of irreversible retinal damage was seen or if patients were considered as having no potential benefit from continuing treatment, further retreatment was normally terminated.

**Table 6.** Guide to retreatment of patients with neovascular AMD after the initial three monthly ranibizumab injections.

| | **Retreatment with intravitreal ranibizumab injection** | |
|---|---|---|
| | Recommended | Not recommended |
| Fundus | Fresh haemorrhage<br>Extension of original lesion | Fibrosis<br>Disciform lesion |
| OCT[a] | Intraretinal or subretinal fluid not resolved or recurrent | Persistent intra- or subretinal or sub-retinal pigment epithelium fluid<br>Evidence of structural retinal damage |
| BCVA[b] | Rapid decrease | Persistently no improvement or a slow decrease |

[a]Optical coherence tomography. [b]Best corrected visual acuity.

If retreatment was decided, patients were treated with a specific as-needed regimen agreed upon in our clinic. This regimen suggested a set of three additional monthly injections when retreatment was decided but if retreatment was not needed, a stepwise increase in the time periods between follow-up visits was recommended (4-6 weeks, 6-8 weeks, 8-10 weeks, etc.). Data were collected on the total number of intravitreal injections over the course of 12 months from the first injection as well as the number of injections per diseased eye.

### 3.2.3 Data analyses

The annual population-based incidence of neovascular AMD in Iceland was established from the number of patients enrolling in the study during 2008 and 2009. The nominator in the annual incidence calculations was the number of patients, not eyes, per year over this 2-year period. The denominator was the number of persons 60 years and older in Iceland at that time. The patients enrolled in 2007 were not included in the determination of the incidence for two reasons. Firstly, not all ophthalmologists in Iceland had started referring patients to treatment as it was still relatively new throughout the world and just became available in March that year in Iceland. Secondly, those ophthalmologists who

had started referring may also have referred the patients diagnosed a few weeks or months earlier, and this accumulated demand could have caused "inflated incidence" in 2007.

In the analysis on the number of intravitreal ranibizumab injections administrated over the course of 12 months, the only data included were from eyes (first and second eye where applicable) of patients who initiated treatment in 2007 and 2008 and had a full 12-month follow-up period. Due to grave economic conditions in Iceland, ranibizumab was abruptly replaced by the much cheaper bevacizumab in early 2010 and therefore a full 12-month follow-up on ranibizumab treatment load was not available for the patients starting treatment in 2009.

# 4 Results

## 4.1 The Retinal Oximetry Study (Papers I-III)

Three studies were performed on retinal oximetry. The first was on a healthy population in order to test and describe the new version of the retinal oximeter and produce a normative control group by analysing various aspects possibly affecting oximetry, such as age and gender (Paper I). The second study further analysed a subgroup of the healthy population for determining various technical aspects of the retinal oximeter (Paper II). The third study was on patients with neovascular AMD in comparison to the healthy control group (Paper III).

### 4.1.1 Oximetry in healthy persons (Papers I and II)

#### *4.1.1.1 Oxygen saturation values*

For the 120 eyes from the 120 healthy persons, the oxygen saturation in retinal arterioles was 92.2±3.7% (mean±standard deviation (SD); 95% confidence interval (CI) 91.5 to 92.9%) and in venules 55.6±6.3% (95% CI 54.4 to 56.7%). The arteriovenous difference was 36.7±5.4% (95% CI 35.7 to 37.6%). All the oxygen saturation values presented here have been adjusted for vessel width as described in chapter 3.1.5. As **Figure 21** shows, the retinal arteriolar oxygen saturation values were normally distributed (D'Agostino and Pearson omnibus normality test $K^2$=5.2; p=0.07), whereas the distribution of venous saturation values was skewed to the left and did not pass a normality test ($K^2$=9.5; p=0.009).

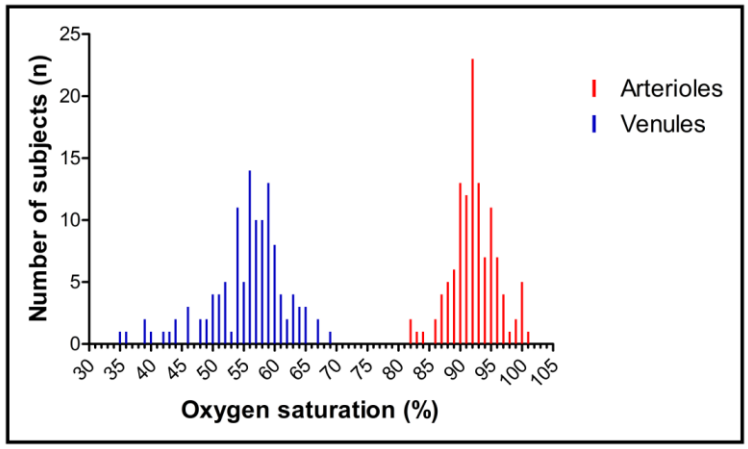


**Figure 21.** Distribution of mean retinal oxygen saturation values in arterioles and venules (n=120). The retinal arteriolar oxygen saturation values were normally distributed (D'Agostino and Pearson omnibus normality test $K^2$=5.2; p=0.07). The distribution of venous saturation was skewed to the left and did not pass a normality test ($K^2$=9.5; p=0.009). Reprinted from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

### 4.1.1.2 Comparing right and left eyes

Both eyes were analysed in 105 participants for determination of reliability of the oximeter (Paper I). As can be see in **Figure 22**, the oxygen saturation of arterioles in right eyes was 92.5±3.6% and in left eyes 92.8±3.4% (paired t-test; p=0.30). In venules, the oxygen saturation in right and left eyes was 56.4±5.7% and 55.6±5.6% (p=0.07), respectively.

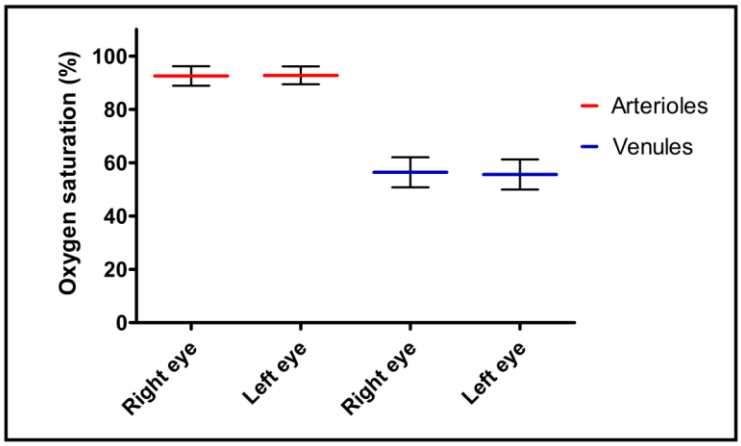


**Figure 22.** Mean arteriolar and venular oxygen saturation (%) in right and left eyes. The error bars show the standard deviation (n=105). Reprinted from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

### 4.1.1.3 Repeatability of measurements

The repeatability of oximetry measurements was determined in the subgroup analysis of 26 healthy subjects (Paper II). **Table 7** shows the results of repeatability measurements of the same retina on two separate images (images 2 and 5 on **Figure 15**), both for an average of the entire fundus and for values from a single retinal vessel (superotemporal arteriole and venule).

**Table 7**. Repeatability of retinal vessel oxygen saturation (%) measurements from 26 eyes in 26 subjects. Each measurement was performed on the same vessel segments in two images from the same visit. Reproduced from Paper II (Invest Ophthalmol Vis Sci, 53(4), 1729-1733, ©2012, with permission of Association for Research in Vision and Ophthalmology).

| | Mean±SD[a] | Mean of differences between repeated (two) measurements[b] | SD of repeated measurements[c] |
|---|---|---|---|
| Arterioles (entire fundus) | 94.1±2.3 | -0.5 | 0.8 |
| Venules (entire fundus) | 64.9±3.3 | -1.1 | 1.3 |
| Main superotemporal arteriole | 93.1±2.8 | -0.3 | 1.0 |
| Main superotemporal venule | 66.7±4.5 | -0.3 | 1.4 |

[a]Mean±SD for the group of 26 individuals was determined from average of the two repeated measurements of each individual.
[b]First image (image 2) minus the second image (image 5).
[c]Standard deviation is derived from a one-way analysis of variance (ANOVA).

More detailed results of repeated measurements of single vessels are shown in **Figure 23**. Saturation was slightly higher in both arterioles (ANOVA; 0.5±0.8%; p=0.017) and venules (1.1±1.3%; p=0.0009) in the latter image in the imaging protocol (image 5 in **Figure 15**) compared to the earlier image (image 2 in **Figure 15**).

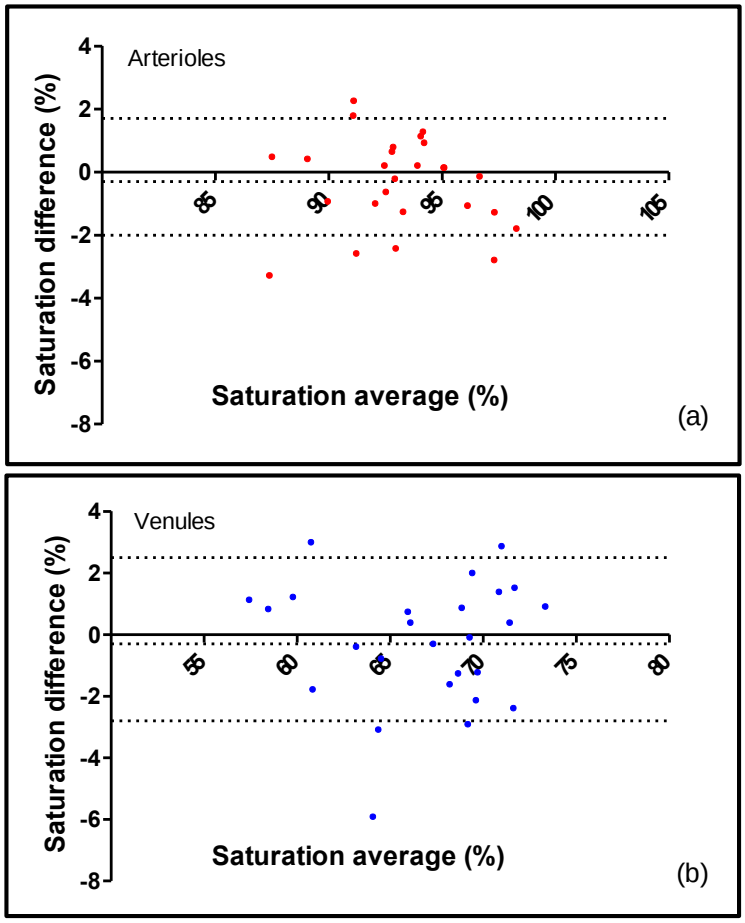


**Figure 23**. Analysis of repeated measurements of retinal vessel oxygen saturation from single vessels. The analysis of (a) arterioles and (b) venules was done on 26 healthy subjects aged 18-30 years. The vertical axis shows the difference between two saturation measurements on the same vessel segment (first image minus the second) and the horizontal axis shows their mean. The broken line in the middle shows the mean difference and the other two broken lines above and below show the ±2 standard deviations for repeated measurements (derived from ANOVA, not the standard deviation of the difference). Reproduced from Paper II (Invest Ophthalmol Vis Sci, 53(4), 1729-1733, ©2012, with permission of Association for Research in Vision and Ophthalmology).

### *4.1.1.4 Topographical variance*

Topographical variance within an image was first analysed in the subgroup study (Paper II) and then expanded for the entire group of healthy individuals (Paper I).

In the subgroup analysis (Paper II), 21 eyes had at least one measureable arteriole in all four quadrants and 19 eyes had at least one measureable venule in all four quadrants. The comparison of the retinal quadrants can be seen in **Table 8**. The results demonstrated that the inferotemporal quadrant had lower saturation measurements compared to the other three quadrants for both arterioles and venules.

**Table 8**. Comparison of retinal vessel oxygen saturation (%) for arterioles (n=21) and venules (n=19) in different retinal quadrants. The table shows the results of Tukey post-tests where the row is subtracted from the column. All numbers are oxygen saturation percentages (mean±SD and 95% confidence interval (CI)). There is a significant variation between the retinal quadrants ($p<0.0001$ by ANOVA) for both arterioles and venules. Asterisk marks statistically significant difference (*$p<0.05$). Reproduced from Paper II (Invest Ophthalmol Vis Sci, 53(4), 1729-1733, ©2012, with permission of Association for Research in Vision and Ophthalmology).

| **Arterioles** | **Superonasal** | **Inferonasal** | **Inferotemporal** |
|---|---|---|---|
| Superotemporal | 2.1±3.7*<br>CI: 0.3 to 3.9 | 1.8±2.3<br>CI: -0.1 to 3.6 | -2.4±3.3*<br>CI: -4.2 to -0.6 |
| Superonasal | | -0.3±3.4<br>CI: -2.1 to 1.5 | -4.5±2.9*<br>CI: -6.3 to -2.7 |
| Inferonasal | | | -4.2±3.3*<br>CI: -6.0 to -2.4 |
| **Venules** | **Superonasal** | **Inferonasal** | **Inferotemporal** |
| Superotemporal | -3.6±4.5<br>CI: -7.6 to 0.5 | -1.3±5.5<br>CI: -5.4 to 2.7 | -7.0±8.0*<br>CI: -11.0 to -2.9 |
| Superonasal | | 2.2±5.4<br>CI: -1.8 to 6.3 | -3.4±8.1<br>CI: -7.4 to 0.6 |
| Inferonasal | | | -5.6±7.4*<br>CI: -9.7 to -1.6 |

In the analysis of the total population of healthy subjects (Paper I), 85 eyes had at least one measureable arteriole and venule in all four quadrants, which allowed the arteriovenous difference to be compared between the quadrants as well. **Table 9** shows the oxygen saturation values for arterioles, venules and arteriovenous difference in each quadrant and **Table 10** shows the statistical comparison of oxygen saturation between the retinal quadrants. The analysis confirmed the results of the subgroup analysis, more specifically that the oxygen saturation values were lowest in the inferotemporal quadrant of both arterioles and venules (**Table 9**) and significantly lower in comparison to the other three quadrants (ANOVA $p<0.0001$; **Table 10**). The reverse was true for the arteriovenous difference, which showed the highest oxygen saturation measurements in the inferotemporal quadrant (**Table 9**) and a significant variation between quadrants (ANOVA $p<0.0001$), although not reaching statistically significance in comparison to each respective quadrant (**Table 10**).

**Table 9**. The retinal vessel oxygen saturation values (%; mean±SD) for arterioles, venules and the arteriovenous (AV) difference in each retinal quadrant (n=85). Measurements in eyes that had at least one measureable arteriole and venule in all four quadrants.

| | Arterioles | Venules | AV difference |
|---|---|---|---|
| Inferotemporal | 88.2±5.9% | 47.4±9.4% | 40.8±11.5% |
| Superotemporal | 90.7±5.3% | 57.4±8.0% | 33.3±8.7% |
| Inferonasal | 93.3±4.9% | 56.5±6.9% | 36.7±7.9% |
| Superonasal | 95.6±6.2% | 56.5±7.1% | 39.1±8.9% |

**Table 10**. Comparison of retinal vessel oxygen saturation (%) in different retinal quadrants for arterioles, venules and arteriovenous (AV) difference. The table shows the results of Tukey post-tests where the row is subtracted from the column (n=85). All numbers are oxygen saturation percentages (mean±SD and 95% confidence interval (CI)). There is a significant variation between quadrants ($p<0.0001$ by ANOVA) for arterioles, venules and AV difference. Asterisks mark statistically significant difference (*$p<0.05$; $^{\dagger}p<0.01$; $^{\ddagger}p<0.001$). Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

| **Arterioles** | **Superonasal** | **Inferonasal** | **Inferotemporal** |
|---|---|---|---|
| Superotemporal | 4.9±6.5‡<br>CI: 3.1 to 6.6 | 2.5±5.9†<br>CI: 0.8 to 4.3 | -2.5±6.5†<br>CI: -4.3 to -0.8 |
| Superonasal | | -2.3±6.0†<br>CI: -4.1 to -0.6 | -7.4±6.8‡<br>CI: -9.1 to -5.6 |
| Inferonasal | | | -5.1±5.6‡<br>CI: -6.8 to -3.3 |
| **Venules** | **Superonasal** | **Inferonasal** | **Inferotemporal** |
| Superotemporal | -1.0±7.4<br>CI: -3.5 to 1.6 | -0.9±8.4<br>CI: -3.4 to 1.6 | -10.0±9.8‡<br>CI: -12.5 to -7.5 |
| Superonasal | | 0.1±8.1<br>CI: -2.5 to 2.6 | -9.0±9.7‡<br>CI: -11.6 to -6.5 |
| Inferonasal | | | -9.1±10.2‡<br>CI: -11.6 to -6.6 |
| **AV difference** | **Superonasal** | **Inferonasal** | **Inferotemporal** |
| Superotemporal | 5.8±8.4‡<br>CI: 2.1 to 9.5 | 3.4±8.9<br>CI: -0.3 to 7.2 | 7.5±11.7‡<br>CI: 3.7 to 11.2 |
| Superonasal | | -2.4±9.3<br>CI: -6.1 to 1.3 | 1.7±12.1<br>CI: -2.1 to 5.4 |
| Inferonasal | | | 4.0±11.7*<br>CI: 0.3 to 7.8 |

### *4.1.1.5 Vessel location in image*

The effects of different location of a vessel within an image was determined in the subgroup analysis (Paper II) by comparing oxygen saturation values from images of different gaze. **Table 11** shows the results of the vessel location analysis.

The vessels below the optic disc, the inferior arcade, had higher retinal oxygen saturation values in the image where the subject was looking down (effects of downgaze in **Figure 20**; optic disc moved up in image 4 in **Figure 15**) and the inferior arcade vessels were closer to the center of the image, compared to the image where the optic disc is in the center and the same vessel arcade is found in the inferior part of the image. This was true for both arterioles (paired t-test; p=0.0004) and venules (p=0.0007). The vessels above the optic disc showed no significant difference between images where the subject looked up (effects of upgaze in **Figure 19**; optic disc moved down in image 3 in **Figure 15**) and the superior arcade vessels were closer to the center of the image, compared to the image with the optic disc in the center, yet a trend towards lower saturation with an upward gaze was seen in the arterioles (p=0.052).

**Table 11**. Changes in retinal vessel oxygen saturation (%) by vessel location within an image. The results show the average change (mean±SD) in oxygen saturation values when measurements from images with optic disc moved down or with optic disc moved up are subtracted from measurements from image with optic disc in the center (n=26). Reproduced from Paper II (Invest Ophthalmol Vis Sci, 53(4), 1729-1733, ©2012, with permission of Association for Research in Vision and Ophthalmology).

| Vessel location | Change in oxygen saturation (mean±SD) | p-value (paired t-test) | 95% CI |
|---|---|---|---|
| Arterioles above optic disc[a] | 0.4±1.1% | 0.052 | -0.9 to 0.0 |
| Venules above optic disc[a] | 0.1±2.2% | 0.87 | -1.0 to 0.8 |
| Arterioles below optic disc[b] | -1.3±1.7% | 0.0004 | 0.7 to 2.0 |
| Venules below optic disc[b] | -1.9±2.4% | 0.0007 | 0.9 to 2.8 |

[a]Oxygen saturation values in image 2 in **Figure 15** subtracted from oxygen saturation values in image 3 in **Figure 15**.
[b]Oxygen saturation values in image 2 in **Figure 15** subtracted from oxygen saturation values in image 4 in **Figure 15**.

#### *4.1.1.6 Age and gender*

The effects of age and gender on retinal oxygen saturation were determined for the entire study population (Paper I), both with a simple linear regression (n=120) and multiple linear regression (n=116).

A simple linear regression of retinal vessel oxygen saturation in relation to age for each gender separately, showed no significant change in arteriolar oxygen saturation with age in either males (slope=-0.04±0.04%/year (mean±standard error of mean (SEM); $r^2$=0.025; 95% CI -0.11 to 0.04; p=0.30) or females (slope=0.03±0.02%/year; $r^2$=0.021; 95% CI -0.02 to 0.08; p=0.23) (**Figure 24a**). However, in venules, a significant decrease in oxygen saturation was noted with increasing age in males (slope=-0.19±0.06%/year; $r^2$=0.19; 95% CI -0.31 to -0.07; p=0.003) and a similar trend in females (slope=-0.07±0.04%/year; $r^2$=0.047; 95% CI -0.14 to -0.01; p=0.068) (**Figure 24a**). The arteriovenous difference was seen to significantly increase with increasing age in both males (slope=0.15±0.05%/year; $r^2$=0.18; 95% CI 0.05 to 0.25; p=0.0043) and females (slope=0.098±0.035%/year; $r^2$=0.10; 95% CI 0.03 to 0.17; p=0.0067) (**Figure 24b**).

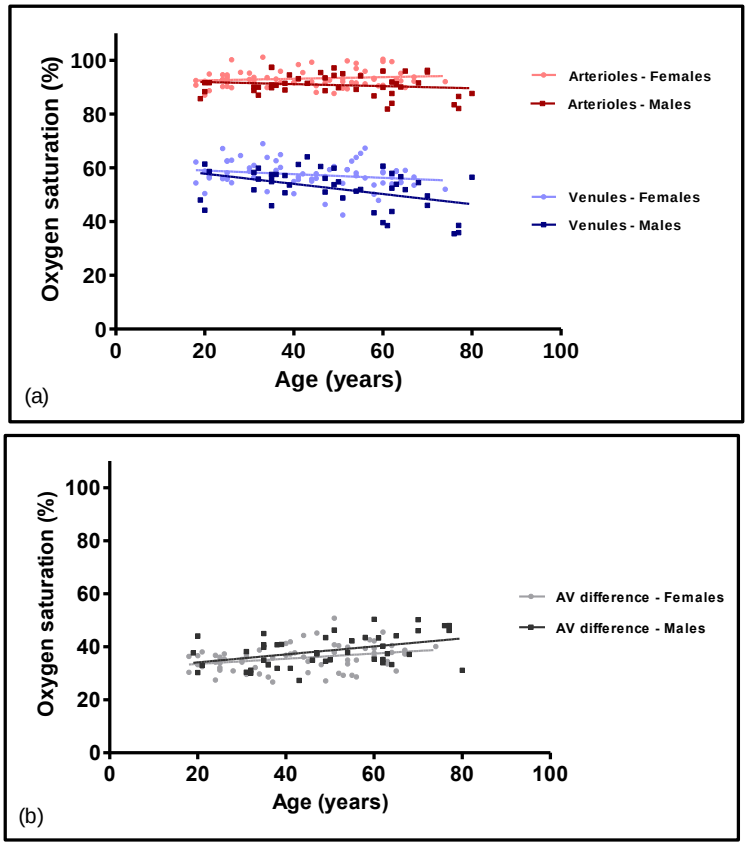


**Figure 24**. Retinal vessel oxygen saturation (%) with age for (a) arterioles and venules and (b) the arteriovenous (AV) difference in males and females. In arterioles, the slope for males was -0.04±0.04%/year (p=0.30) and for females 0.03±0.02%/year (p=0.23). In venules the slope for males was -0.19±0.06%/year (p=0.003) and for females -0.07±0.04%/year (p=0.068). In the AV difference the slope for males was 0.15±0.05%/year (p=0.0043) and for females 0.098±0.035%/year (p=0.0067). Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

The multivariate analysis was performed using multiple linear regression models for arterioles, venules and the arteriovenous difference. The variables for the final models for arterioles, venules and the arteriovenous difference were chosen using a backward selection, although a forward selection was used for testing the preliminary models. **Table 12** shows three preliminary linear regression models as well as the final model for arterioles. The first model shown (A1) was an extension from the simple linear regression model with only age and gender included. However, as the simple linear regression analysis had shown different slopes for males and females, the interaction between age and gender was included in the second model (A2) to test if age had different effects in the two genders. In the third model (A3), all available variables for the subjects were included: age, gender,

current smoking status, finger pulse oximetry and ocular perfusion pressure. The final model for the arterioles (A4) included also the interaction between age and gender as it showed a trend towards significance in arterioles.

**Table 12**. The results of the multivariate analysis for arterioles using multiple linear regression models. The linear regression equation for each model is shown in the heading with $SaO_2$ as retinal vessel oxygen saturation (%) and the coefficients named accordingly (n=116). Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

| **Arterioles**<br>**Variable** | **Estimate** | **Standard Error** | **p-value** |
|---|---|---|---|
| Model A1: $SaO_2 = a_0 + a_1 * A + a_2 * G$<br>Multiple linear regression for age and gender with no interaction between age and gender | | | |
| (Intercept) | 90.7 ($a_0$) | 1.16 | <0.0001 |
| Age (*A*; years) | 0.0012 ($a_1$) | 0.021 | 0.95 |
| Gender (*G*; male=0; female=1) | 2.44 ($a_2$) | 0.69 | 0.0006 |
| Model A2: $SaO_2 = b_0 + b_1 * A + b_2 * G + b_2 * A * G$<br>Multiple linear regression for age and gender with interaction between age and gender | | | |
| (Intercept) | 92.7 ($b_0$) | 1.66 | <0.0001 |
| Age (*A*; years) | -0.038 ($b_1$) | 0.031 | 0.23 |
| Gender (*G*; male=0; female=1) | -0.74 ($b_2$) | 2.07 | 0.72 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | 0.068 ($b_3$) | 0.042 | 0.11 |
| Model A3: $SaO_2 = c_0 + c_1 * A + c_2 * G + c_3 * SS + c_4 * SpO_2 + c_5 * OPP$<br>Multiple linear regression of all variables with no interaction between age and gender | | | |
| (Intercept) | 59.8 ($c_0$) | 27.7 | 0.033 |
| Age (*A*; years) | -0.018 ($c_1$) | 0.023 | 0.45 |
| Gender (*G*; male=0; female=1) | 2.58 ($c_2$) | 0.69 | 0.0003 |
| Current smoking status (*SS*; non-smoker=0, smoker=1) | -1.23 ($c_3$) | 1.02 | 0.23 |
| Finger pulse oximetry ($SpO_2$; %) | 0.28 ($c_4$) | 0.28 | 0.33 |
| Ocular perfusion pressure (*OPP*; mmHg) | 0.097 ($c_5$) | 0.041 | 0.020 |
| Model A4: Final model: $SaO_2 = x_0 + x_1 * A + x_2 * G + x_3 * SS + x_4 * SpO_2 + x_5 * OPP + x_6 * A * G$<br>Multiple linear regression of all variables including interaction between age and gender | | | |
| (Intercept) | 58.8 ($x_0$) | 27.5 | 0.035 |
| Age (*A*; years) | -0.057 ($x_1$) | 0.033 | 0.086 |
| Gender (*G*; male=0; female=1) | -0.66 ($x_2$) | 2.03 | 0.74 |
| Current smoking status (*SS*; non-smoker=0, smoker=1) | -1.30 ($x_3$) | 1.02 | 0.20 |
| Finger pulse oximetry ($SpO_2$; %) | 0.31 ($x_4$) | 0.28 | 0.27 |
| Ocular perfusion pressure (*OPP*; mmHg) | 0.094 ($x_5$) | 0.041 | 0.024 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | 0.069 ($x_6$) | 0.041 | 0.094 |
| Adjusted $R^2$ for models A1, A2, A3 and the final model A4 were 0.086, 0.099, 0.13 and 0.15 | | | |

The simple and multiple linear regession models for arterioles showed corresponding results regarding the effect of age. Like in the simple model (**Figure 24a**), retinal arteriolar oxygen saturation did not change with age after adjusting for gender only (p=0.95; Model A1 in **Table 12**) and this was still true in the final model after adjusting for all the other variables (p=0.086; Model A4 in **Table 12**).

As suggested in the simple linear regression, where there appeared to be some difference between the genders, especially with increasing age, a statistically significant difference could be seen between genders after adjusting solely for age (p=0.0001; Model A1 in **Table 12**). However, this difference disappeared when adjusting for the interaction between age and gender, regardless if the other variables were included (p=0.74; Model A4 in **Table 12**) or not (p=0.72; Model A2 in **Table 12**). The reason for this is that the gender difference is primarily noted in older age groups, as arteriolar oxygen saturation has a tendency to decrease with age in males but not in females (**Figure 24a**).

**Table 13**. The results of the multivariate analysis for venules using multiple linear regression models. The linear regression equation for each model is shown in the heading with $SaO_2$ as retinal vessel oxygen saturation (%) and the coefficients named accordingly (n=116). Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

| **Venules**<br>**Variable** | **Estimate** | **Standard Error** | **p-value** |
|---|---|---|---|
| Model V1: $SaO_2 = a_0 + a_1 * A + a_2 * G$<br>Multiple linear regression for age and gender with no interaction between age and gender | | | |
| (Intercept) | 58.1 ($a_0$) | 1.82 | <0.0001 |
| Age (*A*; years) | -0.12 ($a_1$) | 0.033 | 0.0004 |
| Gender (*G*; male=0; female=1) | 4.4 ($a_2$) | 1.09 | <0.0001 |
| Model V2: $SaO_2 = b_0 + b_1 * A + b_2 * G + b_2 * A * G$<br>Multiple linear regression for age and gender with interaction between age and gender | | | |
| (Intercept) | 61.6 ($b_0$) | 2.60 | <0.0001 |
| Age (*A*; years) | -0.19 ($b_1$) | 0.050 | 0.0003 |
| Gender (*G*; male=0; female=1) | -1.21 ($b_2$) | 3.25 | 0.71 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | 0.12 ($b_3$) | 0.066 | 0.069 |
| Model V3: $SaO_2 = c_0 + c_1 * A + c_2 * G + c_3 * SS + c_4 * SpO_2 + c_5 * OPP$<br>Multiple linear regression of all variables with no interaction between age and gender | | | |
| (Intercept) | -11.0 ($c_0$) | 44.4 | 0.81 |
| Age (*A*; years) | -0.13 ($c_1$) | 0.038 | 0.001 |
| Gender (*G*; male=0; female=1) | 4.43 ($c_2$) | 1.11 | 0.0001 |
| Current smoking status (*SS*; non-smoker=0, smoker=1) | 0.29 ($c_3$) | 1.64 | 0.86 |
| Finger pulse oximetry ($SpO_2$; %) | 0.66 ($c_4$) | 0.45 | 0.15 |
| Ocular perfusion pressure (*OPP*; mmHg) | 0.12 ($c_5$) | 0.066 | 0.065 |
| Model V4: Final model: $SaO_2 = x_0 + x_1 * A + x_2 * G + x_3 * SS + x_4 * SpO_2 + x_5 * OPP + x_6 * A * G$<br>Multiple linear regression of all variables including interaction between age and gender | | | |
| (Intercept) | -12.7 ($x_0$) | 43.9 | 0.77 |
| Age (*A*; years) | -0.20 ($x_1$) | 0.052 | 0.0003 |
| Gender (*G*; male=0; female=1) | -1.32 ($x_2$) | 3.25 | 0.69 |
| Current smoking status (*SS*; non-smoker=0, smoker=1) | 0.17 ($x_3$) | 1.62 | 0.92 |
| Finger pulse oximetry ($SpO_2$; %) | 0.71 ($x_4$) | 0.45 | 0.12 |
| Ocular perfusion pressure (*OPP*; mmHg) | 0.12 ($x_5$) | 0.065 | 0.075 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | 0.12 ($x_6$) | 0.065 | 0.063 |
| Adjusted $R^2$ for models V1, V2, V3 and the final model V4 were 0.23, 0.25, 0.25 and 0.26 | | | |

Ocular perfusion pressure was the only variable that significantly affected the retinal arteriolar oxygen saturation (p=0.024; Model A4 in **Table 12** and the effects will be presented in more detail in chapter 4.1.1.7.

The models for venules are shown in **Table 13**, which includes the three preliminary linear regression models (V1-V3) as well as the final model (V4), which incorporated the interaction between age and gender as it showed a trend towards significance in venules.

As in arterioles, the simple and multiple linear regession models for venules showed corresponding results with regards to age. In the simple model, the retinal venular oxygen saturation decreased significantly with age (**Figure 24a**) and this effect remained in the most simple multiple linear regession model, which only corrected for age and gender (p=0.0003; Model V1 in **Table 13**). In the final model, after adjusting for all the other variables, as well as the interaction between age and gender, the venular oxygen saturation was still seen to decrease with age (p=0.0003; Model V4 in **Table 13**).

The apparent difference between genders seen in the simple linear regression could also be seen in the multiple linear regression model including only age and gender (p<0.0001; Model V1 in **Table 13**, but this difference in venular oxygen saturation between the genders fully disappeared after taking into account the interaction between age and gender, irrespective of whether other variables were included in the model (p=0.71; Model V4 in **Table 13**) or not (p=0.69; Model V2 in **Table 13**). Consequently, this difference in venular oxygen saturation between genders was in fact partly dependent on age like the simple linear regression model suggested, as the lines deviate from each other with increasing age (**Figure 24a**).

The models for arteriovenous difference are shown in **Table 14**, which includes the three preliminary linear regression models (AV1-AV3) as well as the final model (AV4). The final model for arteriovenous difference was different from the final models for arterioles and venules as it did not include the interaction between age and gender since this variable did not show any trend towards significance in the case of the arteriovenous difference.

The arteriovenous difference increased significantly with increasing age in both males and females in the simple model (**Figure 24b**). The multiple linear regression confirmed this effect in all its models (AV1-AV4). In the final model (AV4), the estimated increase in arteriovenous difference for every 10 years is about 1% (p=0.0015; Model AV4 in **Table 14**). The simple linear model suggested a small difference between the genders in the case of the arteriovenous difference (**Figure 24b**) and that was also present in the first multiple linear model (p=0.042; Model AV1 in **Table 14**), but this was not confirmed in the final model including all the other variants, although a trend was seen (p=0.064; Model AV4 in **Table 14**).

**Table 14**. The results of the multivariate analysis for arteriovenous (AV) difference using multiple linear regression models. The linear regression equation for each model is shown in the headings with $SaO_2$ as retinal vessel oxygen saturation (%) and the coefficients named accordingly (n=116). Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

| **Arteriovenous (AV) difference**<br>**Variable** | **Estimate** | **Standard Error** | **p-value** |
|---|---|---|---|
| Model AV1: $SaO_2 = a_0 + a_1 * A + a_2 * G$<br>Multiple linear regression for age and gender with no interaction between age and gender | | | |
| (Intercept) | 32.6 ($a_0$) | 1.60 | <0.0001 |
| Age (*A*; years) | 0.12 ($a_1$) | 0.029 | <0.0001 |
| Gender (*G*; male=0; female=1) | -1.98 ($a_2$) | 0.96 | 0.042 |
| Model AV2: $SaO_2 = b_0 + b_1 * A + b_2 * G + b_2 * A * G$<br>Multiple linear regression for age and gender with interaction between age and gender | | | |
| (Intercept) | 31.1 ($b_0$) | 2.32 | <0.0001 |
| Age (*A*; years) | 0.15 ($b_1$) | 0.045 | 0.001 |
| Gender (*G*; male=0; female=1) | 0.48 ($b_2$) | 2.89 | 0.87 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | -0.053 ($b_3$) | 0.059 | 0.37 |
| Model AV3: $SaO_2 = c_0 + c_1 * A + c_2 * G + c_3 * SS + c_4 * SpO_2 + c_5 * OPP + c_6 * A * G$<br>Multiple linear regression of all variables with interaction between age and gender | | | |
| (Intercept) | 71.5 ($c_0$) | 39.7 | 0.074 |
| Age (*A*; years) | 0.14 ($c_1$) | 0.047 | 0.0039 |
| Gender (*G*; male=0; female=1) | 0.66 ($c_2$) | 2.94 | 0.82 |
| Current smoking status (*SS*; non-smoker=0, smoker=1) | -1.47 ($c_3$) | 1.46 | 0.32 |
| Finger pulse oximetry ($SpO_2$; %) | -0.40 ($c_4$) | 0.41 | 0.33 |
| Ocular perfusion pressure (*OPP*; mmHg) | -0.024 ($c_5$) | 0.059 | 0.69 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | -0.053 ($c_6$) | 0.059 | 0.37 |
| Model AV4: Final model: $SaO_2 = x_0 + x_1 * A + x_2 * G + x_3 * SS + x_4 * SpO_2 + x_5 * OPP$<br>Multiple linear regression of all variables excluding interaction between age and gender | | | |
| (Intercept) | 70.8 ($x_0$) | 39.7 | 0.77 |
| Age (*A*; years) | 0.11 ($x_1$) | 0.034 | 0.0015 |
| Gender (*G*; male=0; female=1) | -1.85 ($x_2$) | 0.99 | 0.064 |
| Current smoking status (*SS*; non-smoker=0, smoker=1) | -1.52 ($x_3$) | 1.46 | 0.30 |
| Finger pulse oximetry ($SpO_2$; %) | -0.38 ($x_4$) | 0.41 | 0.36 |
| Ocular perfusion pressure (*OPP*; mmHg) | -0.026 ($x_5$) | 0.059 | 0.66 |
| Adjusted $R^2$ for models AV1, AV2, AV3 and the final model AV4 were 0.17, 0.17, 0.16 and 0.16 | | | |

### *4.1.1.7 Ocular perfusion pressure*

**Figure 25** shows that ocular perfusion pressure increased with increasing age (slope=0.21±0.044 mmHg/year (mean±SEM); $r^2$=0.16; 95% CI 0.12 to 0.29; p<0.0001). As can be seen in **Table 12** (A4 final model for arterioles) and **Table 13** (V4 final model for venules), every 1 mmHg increase in ocular perfusion pressure, increased the oxygen saturation in arterioles by 0.09±0.04% (mean±SEM; p=0.024) and in venules by 0.12±0.07% (p=0.075).

Ocular perfusion pressure did, however, not have any association with the arteriovenous difference (p=0.66).

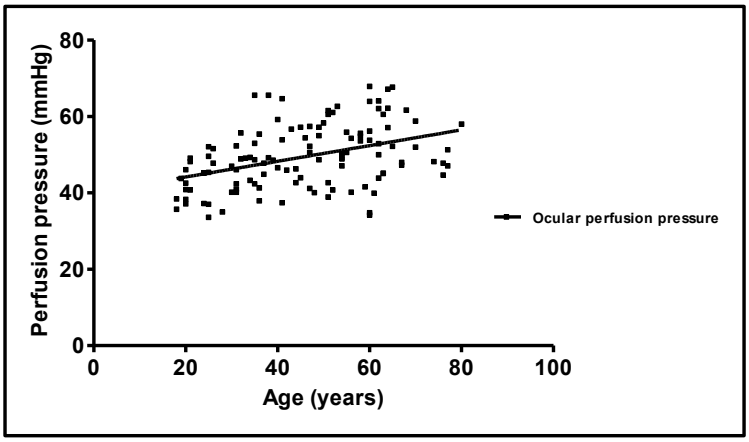


**Figure 25**. Ocular perfusion pressure (mmHg) with increasing age. The slope was 0.21±0.044 mmHg/year (p<0.0001; n=117). Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

As the calculation of ocular perfusion pressure included systolic and diastolic blood pressure as well as intraocular pressure, these variables could not be included independently in the statistical models.

#### *4.1.1.8 Smoking*

Retinal oxygen saturation was not different in current smokers compared to non-smokers in arterioles (**Table 12**; p=0.20), venules (**Table 13**; p=0.92) or the arteriovenous difference (**Table 14**; p=0.30).

#### *4.1.1.9 Systemic oxygen saturation*

No association was noted between finger pulse oxygen saturation and retinal oxygen saturation in arterioles (**Table 12**; p=0.27), venules (**Table 13**; p=0.12) or the arteriovenous difference (**Table 14**; p=0.36).

#### *4.1.1.10 Vessel diameters*

Diameter of retinal arterioles was 10.9±1.0 pixels (mean±SD) and 13.9±1.2 pixels for venules. As seen in **Figure 26**, with increasing age, the retinal vessel diameter decreased in both arterioles (slope=-0.01±0.005 pixels/year (mean±SEM); $r^2$=0.033; 95% CI -0.021 to -0.0002; p=0.046) and venules (slope=-0.01±0.007 pixels/year; $r^2$=0.017; 95% CI -0.023 to 0.004; p=0.16).

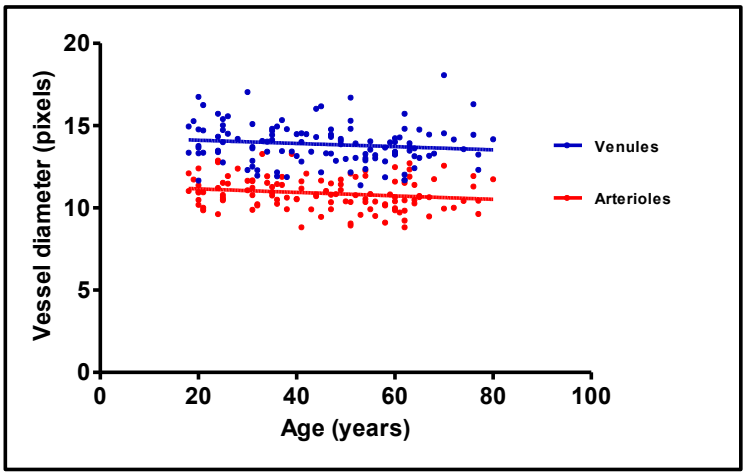


**Figure 26**. Diameter (pixels) of retinal arterioles and venules with increasing age (n=120). For arterioles, the slope was -0.01±0.005 pixel/year (p=0.046). For venules, the slope was -0.01±0.007 pixel/year (p=0.16). Reproduced from Paper I (Invest Ophthalmol Vis Sci, 53(9), 5433-5442, ©2012, with permission of Association for Research in Vision and Ophthalmology).

### 4.1.2 Oximetry in neovascular AMD patients (Paper III)

The oxygen saturation of the AMD patients was 91.5±4.5% (mean±SD) in retinal arterioles, 54.4±9.0% in venules and the arteriovenous difference was 37.1±7.1%. In a crude comparison of the mean oxygen saturation values to the healthy group, there was a trend towards lower saturation in arterioles in AMD patients (p=0.058) but the venular oxygen saturation was significantly lower in AMD patients (p=0.0006) and the arteriovenous difference was significantly higher in AMD patients in comparison to the healthy persons (p=0.004).

**Figure 27a** presents the results of the simple linear regression of arteriolar and venular oxygen saturation in relation to age, in neovascular AMD patients and healthy controls. Comparable results for arteriovenous difference are shown in **Figure 27b**. In arterioles, there was a discrete and insignificant increase in oxygen saturation with age in AMD patients (slope=0.16±0.10%/year; $r^2$=0.052; 95% CI -0.045 to 0.36; p=0.13) but no change was seen in healthy controls (slope=-0.0070±0.021%/year; $r^2$=0.001; 95% CI -0.048 to 0.034; p=0.73). In a comparison between the two groups, a trend towards difference was seen, although significance levels were not reached (ANCOVA; p=0.075).

Conversely, in venules, there was a significant increase in oxygen saturation with age in AMD patients (slope=0.45±0.19%/year; $r^2$=0.11; 95% CI 0.057 to 0.84; p=0.026) and a significant decrease in oxygen saturation with age in healthy persons (slope=-0.13±0.03%/year; $r^2$=0.11; 95% CI -0.19 to -0.06; p=0.0002). The difference in venular oxygen saturation between the AMD patients and the healthy controls was highly significant (ANCOVA; p=0.0003).

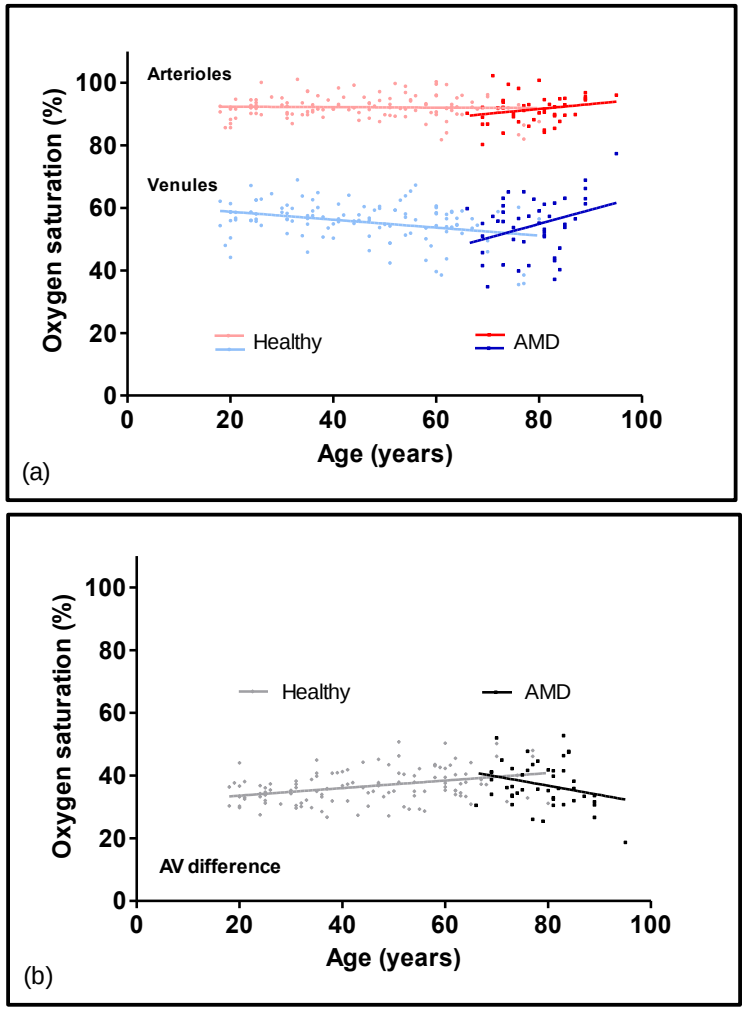


**Figure 27**. Retinal vessel oxygen saturation (%) in arterioles, venules and the arteriovenous (AV) difference for healthy controls aged 18-80 years (n=120) and neovascular AMD patients aged 66-95 years (n=46). (a) The slope for arteriolar oxygen saturation for AMD patients was 0.16±0.10%/year (p=0.13) and for healthy persons -0.0070±0.021%/year (p=0.73). The difference in slopes for arterioles between healthy and AMD patients was not significant (p=0.075). The slope for venular oxygen saturation for AMD patients was 0.45±0.19%/year (p=0.026) and for healthy persons -0.13±0.03%/year (p=0.0002). The difference in slopes for venules between healthy and AMD patients was significant (p=0.0003). (b) The slope for the AV difference for the AMD patients was -0.29±0.16%/year (p=0.065) and for the healthy controls 0.12±0.03%/year (p<0.0001). The slopes for AV difference are significantly different between healthy and AMD patients (p=0.0017). Reproduced from Paper III (Acta Ophthalmol, 92(1), 27-33, ©2014, with permission of John Wiley & Sons).

The same was true for the arteriovenous difference of oxygen saturation. In AMD patients, there was a decrease in oxygen saturation with age, although

not reaching the significance level (slope=-0.29±0.16%/year; $r^2$=0.075; 95% CI -0.61 to 0.020; p=0.065). In the healthy controls, there was a significant increase in oxygen saturation with age (slope=0.12±0.03%/year; $r^2$=0.14; 95% CI 0.065 to 0.18; p<0.0001). The comparison of arteriovenous difference between the two groups showed a significant difference (ANCOVA; p=0.0017).

**Table 15**. The results of the multiple linear regression analysis for arterioles, venules and arteriovenous (AV) difference in the comparison between patients with neovascular AMD (n=42) and the healthy control group (n=117). The final linear regression equations with the selected variables for the three models are presented in the headings for each model with $SaO_2$ as retinal vessel oxygen saturation (%) and the coefficients named accordingly. Reproduced from Paper III (Acta Ophthalmol, 92(1), 27-33, ©2014, with permission of John Wiley & Sons).

| Variable | Estimate | Standard Error | p-value |
|---|---|---|---|
| Arterioles: $SaO_2 = x_0 + x_1*SG + x_2*A + x_3*G + x_4*SS + x_5*SpO_2 + x_6*SP + x_7*A*G + x_7*A*SG$ | | | |
| (Intercept) | 45.2 ($x_0$) | 23.2 | 0.054 |
| Study group (*SG*; healthy=0; AMD=1) | -9.89 ($x_1$) | 6.92 | 0.16 |
| Age (*A*; years) | -0.029 ($x_2$) | 0.031 | 0.35 |
| Gender (*G*; male=0; female=1) | 0.054 ($x_3$) | 1.87 | 0.98 |
| Current smoking status (*SS*; non-smoker=0, smoker=1) | -1.63 ($x_4$) | 0.87 | 0.063 |
| Finger pulse oximetry ($SpO_2$; %) | 0.45 ($x_5$) | 0.24 | 0.068 |
| Systolic blood pressure (*SP*; mmHg) | 0.033 ($x_6$) | 0.018 | 0.077 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | 0.051 ($x_7$) | 0.033 | 0.12 |
| Interaction Age:Study Group (*A*SG*; years; healthy=0; AMD=1) | 0.11 ($x_8$) | 0.089 | 0.21 |
| Venules: $SaO_2 = y_0 + y_1*SG + y_2*A + y_3*G + y_4*SpO_2 + y_5*SP + y_6*A*G + y_7*A*SG$ | | | |
| (Intercept) | -24.7 ($y_0$) | 39.5 | 0.53 |
| Study group (*SG*; healthy=0; AMD=1) | -42.1 ($y_1$) | 11.4 | 0.0003 |
| Age (*A*; years) | -0.16 ($y_2$) | 0.052 | 0.0017 |
| Gender (*G*; male=0; female=1) | 0.35 ($y_3$) | 3.18 | 0.91 |
| Finger pulse oximetry ($SpO_2$; %) | 0.80 ($y_4$) | 0.41 | 0.053 |
| Systolic blood pressure (*SP*; mmHg) | 0.060 ($y_5$) | 0.031 | 0.057 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | 0.089 ($y_6$) | 0.055 | 0.11 |
| Interaction Age:Study Group (*A*SG*; years; healthy=0; AMD=1) | 0.56 ($y_7$) | 0.15 | 0.0002 |
| AV difference: $SaO_2 = z_0 + z_1*SG + z_2*A + z_3*G + z_4*A*G + z_5*A*SG$ | | | |
| (Intercept) | 31.1 ($z_0$) | 2.4 | <0.0001 |
| Study group (*SG*; healthy=0; AMD=1) | 28.6 ($z_1$) | 10.2 | 0.0056 |
| Age (*A*; years) | 0.15 ($z_2$) | 0.044 | 0.0010 |
| Gender (*G*; male=0; female=1) | 0.073 ($z_3$) | 2.76 | 0.98 |
| Interaction Age:Gender (*A*G*; years; male=0; female=1) | -0.044 ($z_4$) | 0.049 | 0.37 |
| Interaction Age:Study Group (*A*SG*; years; healthy=0; AMD=1) | -0.41 ($z_5$) | 0.13 | 0.0025 |
| Adjusted $R^2$ for the arteriolar, venular and AV difference models were 0.14, 0.25 and 0.14 | | | |

For confirmation that the suggested difference between the AMD patients and the healthy controls seen in the simple model was not a result of possible confounding variables between the two groups, multiple linear regression models were made for arterioles, venules and the arteriovenous difference. Backward selection of variables was used to build the final models seen in **Table 15**, from the available variables listed in chapter 3.1.7, but forward selection led to the same final models. The final models for arterioles, venules and the arteriovenous difference were in all cases made to include both the interaction between age and gender and the interaction between age and study group. This was decided because of the established interaction between age and gender in the healthy group as well as the suggested interaction between age and study group seen in the simple linear regression model in **Figure 27**. The final models for arterioles, venules and the arteriovenous difference can be seen within **Table 15**.

In arterioles, the multiple linear regression analysis was consistent with the simple model and showed no difference between the AMD patients and the healthy controls (p=0.16). Hence, the arteriolar oxygen saturation was not affected by any of the variables tested. Moreover, the relationship with age was also not different between the two groups (p=0.21).

In venules, consistency was seen between the multiple linear regression and the simple model. The multivariate analysis showed a significant difference between the AMD patients and the healthy population, with higher oxygen saturation in venules in AMD patients (after age 76 years). More specifically, the coefficient for the interaction of AMD and age ($y_7$ in **Table 15**) was 0.56 percentage points per year (p=0.0002), while the coefficient for age alone ($y_2$ in **Table 15**) was -0.16 percentage points per year (p=0.0017). Venular oxygen saturation, thereby, decreases by 0.16 percentage points for each year of age in healthy persons, while in AMD patients, the venular oxygen saturation increases by 0.40 percentage points for each year of age (0.56-0.16=0.40). Moreover, the coefficient for having neovascular AMD ($y_1$ in **Table 15**) was -42.1 percentage points (p=0.0003), which means that an 80-year old AMD patient would be expected to have 2.7% higher oxygen saturation in retinal venules than a healthy person at the same age (0.56*80-42.1=2.7).

The simple and multiple linear regression models for the arteriovenous difference are also compatible, suggesting a decrease in arteriovenous difference with age in AMD compared with an increase in healthy persons (**Figure 27**, **Table 15**). An AMD patient 70 years and older will be expected to have smaller arteriovenous difference than a healthy person at the same age. At 80 years of age, the arteriovenous difference in an AMD patient is 4.2% less than in a healthy individual (see further in **Table 15**).

## 4.2 The Epidemiological Study on AMD (Paper IV)

### 4.2.1 Annual incidence of neovascular AMD

In total, 293 persons 60 years and older were newly diagnosed with neovascular AMD in at least one eye during 2008 and 2009 in Iceland. At the same time, a total of 50 999 persons were found in this age range in Iceland, corresponding to an annual incidence of 0.29% in that age-group. With advancing age, the incidence of neovascular AMD was shown to increase (see **Figure 28**).

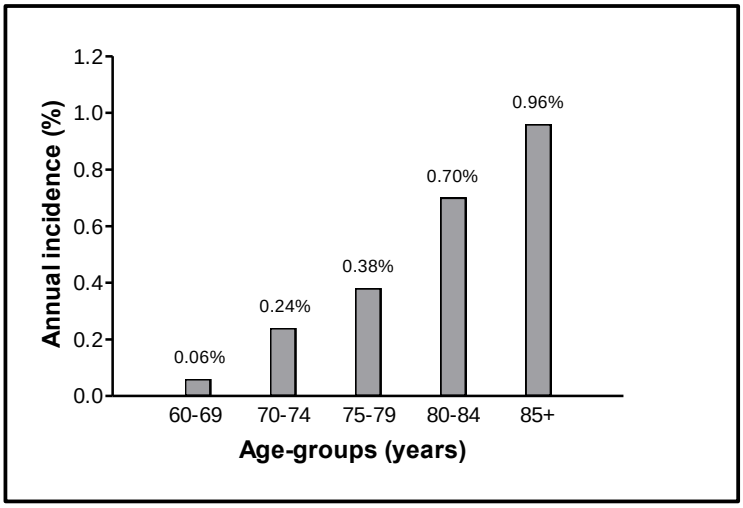


**Figure 28.** Annual incidence of neovascular AMD in Iceland by age-groups (n=293). Reproduced from Paper IV (Br J Ophthalmol, 96(3), 444-447, ©2012, with permission of BMJ Publishing Group Ltd).

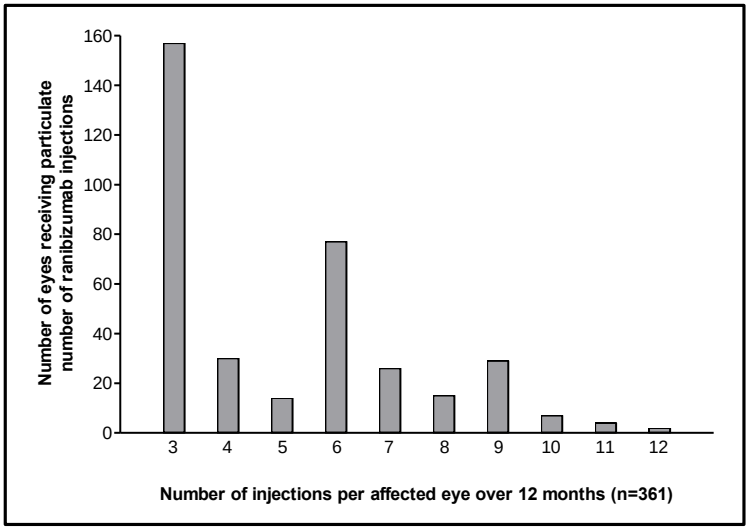


**Figure 29.** Number of ranibizumab intravitreal injections for each eye with neovascular AMD during the first 12 months of treatment (n=361). Reproduced from Paper IV (Br J Ophthalmol, 96(3), 444-447, ©2012, with permission of BMJ Publishing Group Ltd).

### 4.2.2 Number of intravitreal injections over 12 months

Each affected eye enrolled in the study during 2007 and 2008 (n=361) received a mean of 5.0±2.3 (median 4, range 3-12) intravitreal ranibizumab injections over the first 12 months from initiation of treatment. In every case, three injections were mandated by the protocol and given monthly during the first three months of study enrollment. The total number of intravitreal ranibizumab injections administered per affected eye through the first 12 months, can be seen in **Figure 29**.

The average number of intravitreal ranibizumab injections for neovascular AMD was 1213 per year. With this rate of intravitreal injections, it can therefore be expected that every year approximately 2400 intravitreal ranibizumab injections would be required for treatment of neovascular AMD per 100 000 persons aged 60 years and older in Iceland and comparable Caucasian populations.

# 5 Discussion

The results of this thesis suggest that retinal vessel oxygen saturation is abnormal in patients with neovascular age-related macular degeneration (AMD). The new version of the dual-wavelength Oxymap T1 retinal oximeter used in these studies is reliable and yields repeatable results.

Neovascular AMD is one of the most common causes of visual impairment and blindness among older populations in industrialised nations and the results confirmed that the annual incidence of neovascular AMD in Iceland is comparable to other Caucasian populations.

The major findings of the retinal oximetry studies and the AMD incidence study are discussed separately below.

## 5.1 The Retinal Oximetry Study (Papers I-III)

### 5.1.1 Oximetry in healthy persons (Papers I-II)

The two retinal oximetry studies on healthy individuals were performed to test the latest version of the Oxymap T1 retinal oximeter and the Oxymap Analyser software. As with all novel technologies and investigational devices, the oximeter needs to be tested for reliability and technical limitations. Moreover, as the oximeter is intended for studying diseases of the eye, it is important to establish a normative database of retinal vessel oxygen saturation values from healthy individuals by determining the effects of age, gender, systemic oxygenation and blood pressure.

The previous prototype of the Oxymap oximeter had been shown to give sensitive and reliable measurements (Hardarson et al., 2006). Our studies confirmed that the Oxymap T1 retinal oximeter is reliable and gives relatively stable results in a healthy Caucasian population. No difference in oxygen saturation was seen between right and left eyes in our study. The measured oxygen saturation was on average 92.2±3.7% in retinal arterioles and 55.6±6.3%% in retinal venules. These saturation values are comparable to most other studies on retinal oximetry in healthy persons. However, slight differences can be seen among different research groups and different versions of the oximeter. Those variations can usually be accounted for by differences in the retinal oximeter hardware, the analysis software, the analysis methods as well as in the calibration of the oximeter but the calibration of the oximeter is crucial for comparison of oxygen saturation values between different studies.

The calibration of the oximeter used in this study depends on mean oxygen saturation values of retinal vessels in healthy individuals measured with a device designed by Schweitzer et al. (1999), which was calibrated by measuring oxygen saturation in whole blood samples *in vitro*. We made the assumption that their mean retinal vessel oxygen saturation values were as accurate as possible. Our optical density ratios for a similar group of healthy individuals were, therefore, matched to these oxygen saturation values, thus obtaining the values for the calibration constants (*a* and *b* in Equation 7, see chapter 1.3.2) used in our study.

The standard deviations of our oxygen saturation measurements of retinal vessels were 3.7% for arterioles and 6.3% for venules for the whole group of healthy individuals aged 18-80 years. These standard deviations are comparable and even slightly less than in the previously mentioned study by Schweizer et al. (1999), where the standard deviation in arterioles was 4.1% and in venules 9.9%. In a more recent study by Jani et al. (2014), in which the Oxymap T1 retinal oximeter was also used like in our study, the standard deviations were 4.3% in arterioles and 7.1% in venules. The slightly lower standard deviations in our study group can possibly be explained in part by the more homogenous group in our study, as we included only Caucasian persons, whereas in the study by Jani et al. (2014) persons of different ethnicity were included.

In order to standardise our study procedures and analyses, the oximetry images were taken by the same person in a predetermined order and a detailed protocol for the analysis was made. For the purpose of evaluating the mean retinal vessel oxygen saturation for each eye, the data from all the individually measured vessel segments in each eye was averaged without regards to their diameter and thus blood flow. Obviously, there are some limitations to this method with all the vessels contributing equally to the mean. Performing a weighted average might have been an option to account for this imbalance but at the time of the studies no consensus on what was the most appropriate approach to adjust the data was found, therefore the decision to use a simple average was made. Recently, our study group has suggested an appropriate method of weighing the average oxygen saturation measurements (Hardarson et al., 2013b) and some researches have already implemented that method (Yip et al., 2014) but it remains to be answered if that method will result in more accurate measurement values.

#### *5.1.1.1 Repeatability of measurements*

The intra-observer standard deviation of average oxygen saturation values of the retina between repeated measurements on two separate images (images 2 and 5 in **Figure 15**) was 0.8% in arterioles and 1.3% in venules. The standard deviation for repeated measurements of single vessels (main superotemporal vessel) was 1.0% in arterioles and 1.4% in venules.

Previously, Hardarson et al. (2006) demonstrated with an earlier version of the Oxymap oximeter standard deviations for repeated measurements of single vessels of 3.7% for arterioles and 5.3% for venules. Therefore, the current Oxymap oximeter has good repeatability, somewhat better than the previous prototype of the Oxymap oximeter. Hammer et al. (2008) presented variability for averaged fundus measurements with their Imedos oximeter resulting in a mean standard deviation of 2.52% for arterioles and 3.25% for venules. Recently, Man et al. (2013) analysed again the repeatability of the Imedos oximeter system and found the intra-observer standard deviation of averaged oxygen saturation to be 0.56% for arterioles and 0.55% for venules, which is somewhat better than our results. However, standard deviations of repeated measurements of approximately 1%, as achieved in our study, are considered sufficient for most clinical measurements.

It is important to note that the last image of the imaging protocol (image 5 in **Figure 15**), in which the optic nerve was in the centre, consistently showed slightly but significantly higher mean saturation than an earlier image taken in the same manner with the optic nerve in the centre (image 2 in **Figure 15**). This difference was less than 1% and it is unlikely that it will have an effect in most clinical research. The reason for the higher saturation in the latter image is unclear, but it is possible that it is related to the effects and numbers of flashes, though this has not been studied. Heitmar and Cubbidge (2013) showed that with increasing intensity of the flash, the measured oxygen saturation values increased significantly in both retinal arterioles and venules, although significant changes were only seen at flash intensities above 76 Ws. They concluded and recommended flash settings between 27 to 76 Ws. In our study the flash was set at 50 Ws, so the intensity of the flash was within these optimal limits but the effects of multiple flashes in a row, like occurred in our patients, is still undetermined.

#### *5.1.1.2 Topographical variance*

The topographical variability in the measurement within the same image was first anlysed in the smaller subgroup study but then expanded in the larger study for confirmation. In both studies, a significant difference was seen in measured oxygen saturation between the retinal quadrants within the same image. The inferotemporal quadrant showed the significantly lowest oxygen saturation values in arterioles and venules, even up to 10 percentage points lower saturation. Consequently, the arteriovenous difference was highest in the inferotemporal quadrant. Recently, Jani et al. (2014) showed comparable differences in oxygen saturation between different quadrants, with oxygen saturation of the inferotemporal quadrant significantly lowest in both arterioles and venules and similarly, the arteriovenous difference significantly highest in the inferotemporal quadrant. In the recent paper by Yip et al. (2014), the measured oxygen saturation in arterioles and venules was also lowest in the inferotemporal

quadrant and the arteriovenous difference highest although no statistical analysis was included for determination if the differences between the quadrants were significant. Shahidi et al. (2013) studied oxygen saturation in superotemporal and inferotemporal arterioles and venules in healthy young persons using non-flash hyperspectral retinal camera. Despite the different optical approach, they found comparable topographical variance with measured oxygen saturation values of arterioles and venules significantly lower in the inferotemporal quadrant compared to the superotemporal quadrant.

The reason for this topographical variance is still unclear, but it must be considered likely that it is due to an optical artefact. The inferior parts of the images tend to have increased shadows, which likely cause a decrease in the contrast of the vessels in comparison to the background. This would, in turn, affect the evaluation of the optical densities, which are used to calculate the oxygen saturation values. The change in measured oxygen saturation with different vessel location in an image (discussed in next chapter 5.1.1.3) further suggests that this variance is, at least partially, a technical issue. However, this remains to be determined. Patel et al. (2013) evaluated the effects of light scattering on measured retinal vessel oxygen saturation values using an artificial light scatter model. They came to the conclusion that simulated light scatter does indeed affect the measured oxygen saturation values, and possibly in part due to the loss of contrast at the border of the vessel and surrounding tissue. Nevertheless, this does not by any means confirm that the topographical variance seen in our study, as well as in other studies, is due to an optical artefact. Further studies are warranted to exclude a physiological origin of this topographical variance in retinal quadrants.

Hilton et al. (2003) have shown that the inferior part of the retina is more susceptible to insult during hypercapnia, which suggests some topographical variations in retinal oxygenation. Moreover, topographical variations have also been shown in a study by Garhöfer et al. (2012) on retinal blood flow using bidirectional laser Doppler velocimetry in young healthy males. They reported highest retinal blood flow in the inferotemporal quadrant followed by the superotemporal quadrant and then the two nasal quadrants for both retinal arterioles and venules. Interestingly, their blood flow measurements in arterioles were inversely related to our arteriolar oxygen saturation measurements, with the highest blood flow in the quadrant with the lowest saturation. This could point to some regional variations in the oxygen metabolism, although beforehand, it would make more sense that the oxygen saturation would be highest where the blood flow would be greatest, as that would presumably cause less oxygen diffusion from the walls of the arterioles. Regardless of the cause of this difference, it can have notable impact on clinical research on retinal oxygen metabolism and undoubtedly warrants further studies. Moreover, this underlines to the importance of standardisation of retinal oximetry images, the same parts of the retina should be compared.

#### *5.1.1.3 Vessel location in image*

The oxygen saturation in arterioles and venules located in the inferior half of the image changed significantly when the same vessels were compared in images taken at different angles of gaze. Downwards gaze of an eye, which moves a vessel segment up in the image frame, led to higher measured oxygen saturation in those vessel segments. The effect of gaze might, therefore, be related to the variation between quadrants (discussed in the previous chapter) and possibly resulting from optical or illumination aberrations, which are yet to be characterised. Optical artefacts are possibly not the entire explanation as the measured oxygen saturation changed only a couple of percentage points with the movement of the vessel within the image, while the difference in measured oxygen saturation between the inferior and superior quadrants, especially in the temporal region, was several percentage points. This further emphasizes the importance of standardisation of retinal oximetry imaging technique and image analysis with similar images being compared.

#### *5.1.1.4 Retinal oxygen saturation with age and gender*

In healthy persons aged 18 to 80 years, retinal arteriolar oxygen saturation values are stable according to a simple linear regression. However, the measured oxygen saturation in retinal venules decreased significantly with age in males and there was a similar trend in females. For every 10 years of life, the change in saturation was 2% for males and nearly 1% for females. Multiple linear regression confirmed significant decrease in the venular oxygen saturation with age after adjusting for the effects of gender as well as other confounding factors. There is no apparent explanation for this decline in venular oxygen saturation with age but Jani et al. (2014) found the same significant decrease in oxygen saturation of retinal venules with increasing age in a smaller multi-ethnic cohort. Moreover, in their study, a significant decrease in arteriolar oxygen saturation was also seen, whereas the arteriolar oxygen saturation was relatively stable in the multiple linear regression in our study, although a trend towards a decrease with increased age was seen.

It must be considered likely that the decline in venular oxygen saturation with age is due to an artefact. For example, gradual cataract and worse pupil dilation accompanying increasing age could result in worse quality of images as less light might be reaching the retina and the light that gets through might be more scattered. All images were screened before analysis and those with poor quality excluded. However, images of sufficient quality were included even though some signs of early cataract existed. More recently, our research group has developed a software tool that automatically grades the images on the scale of 0 to 1 according to quality of the images based on assessment of focus and contrast (Hardarson et al., 2015). However, at the time of the study,

this software was not available and the possible effects of successively increasing cataract on retinal oximetry unknown.

The fact that similar decline in oxygen saturation was not seen in arteriolar oxygen saturation could possibly be explained by the physiological differences between arterioles and venules. In the previously mentioned study by Patel et al. (2013), the Imedos oximeter was used to evaluate the effects of light scattering from an artificial light scatter model in healthy young persons. This model should in part imitate the effect of cataract on retinal oximetry. Their results suggested that increased light scatter from increasingly more condensed simulated cataract caused an increase in the optical density ratios in both arterioles and venules, although the effect was different on arterioles in comparison to venules. They concluded that light scatter, which can be expected from cataracts, is likely to affect measurements of optical density ratios on both arterioles and venules as well as measurements of the vessel diameters, thereby affecting the calculations of oxygen saturation. As the Imedos oximeter uses a different optical approach and other wavelengths, in comparison to the Oxymap oximeter, cataract or decreased image quality of other causes can possibly give different effects with the two different techniques. Our study group has now presented preliminary results of retinal vessel oxygen saturation in patients with established cataract who were scheduled for cataract surgery of at least one eye (Hardarson et al., 2014). The results showed that the measured oxygen saturation of both arterioles and venules was lower in the eye with more cataract and worse image quality as compared to the oxygen saturation of the eye with better image quality due to less cataract (measured by Nidek EAS-1000 and Pentacam). These results suggest that the decrease in measured venular oxygen saturation with increasing age may possibly be due to increasing cataract. However, further clinical studies on the effects of cataract, for instance before and after cataract surgery, are clearly needed.

In the study by Patel et al. (2013), the diameter of both arterioles and venules generally increased with increased simulated cataract, although the increase in diameter was not entirely linearly increased. It must be kept in mind that their results were independent of age as all participants were young healthy persons. Leung et al. (2003) has shown, that retinal vessel diameter decreases linearly with age, which was also the case in our study, where diameter of both arterioles and venules decreased slightly with age. Moreover, Hammer et al. (2008) reported that narrower retinal vessels have higher measurements of oxygen saturation and because of that, our oxygen saturation measurements were corrected for vessel diameter. According to this, we believed it was unlikely that changes in vessel diameter with increasing age were the causative factor for the decline in venular oxygen saturation with age, as we would rather expect the opposite effect. There is, of course, the possibility that we might have overcorrected for the vessel

diameter. We, however, consider it unlikely as we have previously shown that the relationship between vessel diameter and oxygen saturation was still present after comparable correction for vessel diameter (Gottfredsdottir et al., 2011). Moreover, we have considered it more accurate to perform the vessel diameter correction by determining a correction factor from the measured oxygen saturation values before and after branching of the vessels (as described in detail in chapter 3.1.5) since we believe there might actually be a real relationship between the oxygen saturation and vessel diameter. We have, therefore, decided not to compensate entirely for the vessel diameter by making the oxygen saturation values entirely diameter-independent as presented by Hammer et al. (2008).

The decrease in venular oxygen saturation with age could possibly be of physiological origin. It is interesting to speculate whether males actually deteriorate faster than females as the genders do have a significantly different life expectancy; in Iceland women can expect to live approximately 4 years longer compared to men (Statistical Bureau of Iceland, 2010). Whatever the reason for this change in retinal venular oxygen saturation with age and gender is, it emphasizes the fact that these results must be taken into account in clinical research when patient groups are compared to healthy control groups and stresses again the importance of standardisation; it is necessary to adjust for age and gender.

As the study includes relatively few individuals aged 70 years and older, this observation requires further evaluation with more normative data in healthy individuals over 70 years of age. This will be especially important for the study of age-related eye diseases in this age group. However, it is challenging to find healthy individuals in this age range as many at that age already have systemic and/or eye diseases as well as lens opacities that reduce the retinal image quality.

Moreover, the vitreous humour is composed of gel-like material that has been shown to undergo a process of age-related liquefaction in humans (Los et al., 2003). Vitreous liquefaction is generally a precursor to the process of posterior vitreous detachment (for review see Le Goff and Bishop, 2008), which has been shown to be more common with increased age (Itakura and Kishi, 2013). As discussed in chapter 1.6.3, the low viscosity fluid collecting behind posterior vitreous detachments has much higher diffusion coefficients than the viscous vitreous gel. Theoretically, one could assume that more oxygen would be diffusing from the retina into the vitreous in persons with posterior vitreous detachment as opposed to those with vitreous adhesion, thereby decreasing oxygen saturation in venules. Since posterior vitreous detachment becomes more common with increasing age, it is interesting to speculate if that is one of the reasons why the venular oxygen saturation decreased with age. It could be argued that removing the vitreous with pars

plana vitrectomy might similarly cause increased diffusion of oxygen into the vitreous cavity, especially since the vitreous has been shown to be an oxygen consumer in an ascorbate-dependent manner (Shui et al., 2009). Now Šín et al. (2014) and Lim et al. (2014) have measured retinal vessel oxygen saturation in patients before and after pars plana vitrectomy for various macular conditions. Both studies showed significantly increased venular oxygen saturation after vitrectomy (Lim et al., 2014; Šín et al., 2014) and the study by Lim et al. even showed significantly increased arteriolar saturation and decreased arteriovenous difference after surgery. However, these two studies were executed in quite different ways. In the study by Šín et al. (2014), only one arteriole and one venule were analysed (the ones in inferotemporal quadrant if available, otherwise the ones in the superotemporal quadrant), whereas in the study by Lim et al. (2014), the mean oxygen saturation of the entire fundus was analysed. Moreover, in the study by Šín et al., half of the patients were phakic and the other half pseudophakic. Since vitrectomy is known to accelerate the progression of cataract formation (Holekamp et al., 2005), the measurements of oxygen saturation in the phakic patients could possibly be affected after vitrectomy, although in this study no differences appeared to be found. In the study by Lim et al. (2014) all patients underwent a combined vitrectomy and cataract operation with phacoemulsification technique. Therefore, the increase in arteriolar as well as venular oxygen saturation could possibly be explained by better image quality after surgery due to decreased opacity of the lens, but as discussed previously in this chapter, Hardarson et al. (2014) have found higher oxygen saturation in both arterioles and venules in eyes with less cataract in comparison to their other eye with more cataract.

If blood flow is assumed to be constant, changes in arteriovenous difference in saturation will reflect oxygen extraction from the retinal vessels. Moreover, the arteriovenous difference can be of value as an inner control of the data since artefacts from various causes would be expected, at least beforehand, to have similar effects on arteriolar and venular measurements. Therefore, artefacts could be expected to have a tendency to cancel out in calculations of the arteriovenous difference. However, preliminary data from our research group, regarding the effects of worse image quality on measured oxygen saturation, show that measurements of oxygen saturation in venules tend to be more affected than in arterioles (Hardarson et al., 2015). Consequently, this must be interpreted with caution.

The present study showed that arteriovenous difference increased significantly with age in both males and females. For every 10 years of increasing age, the arteriovenous difference increased by approximately 1.5% in males and 1% in females. This might imply that with age, proportionally more oxygen is extracted from each milliliter blood from the retinal vessels. However, retinal blood flow was not measured in this study and therefore, all speculations regarding

oxygen extraction are difficult. We did measure the retinal vessel diameter, which was seen to decrease with age. That, by itself, causes increased resistance to blood flow and might suggest decreased retinal blood flow with age. However, the ocular perfusion pressure was seen to increase with age in our study and that might to some extent compensate for the decrease in vessel diameter by increasing the blood flow. As published data on retinal blood flow in relation to age is conflicting and retinal blood flow was not measured in our study, the overall effect of age on oxygen extraction from the retinal circulation is difficult to determine, requiring further studies on this matter.

#### *5.1.1.5 Retinal oxygen saturation and systemic oxygenation*

After adjusting for age and other confounding factors, an increase in the ocular perfusion pressure was correlated with an increase in arteriolar oxygen saturation and the same trend was seen in venules as well. A possible explanation for this is that higher ocular perfusion pressure may cause higher blood flow and blood flow velocity, resulting in greater amounts of more oxygenated blood at every point.

Systemic oxygen saturation measured from finger pulse oximeter and retinal oxygen saturation measurements are expected to be correlated, with an increase in systemic oxygenation resulting in an increase in retinal oxygenation, similarly as seen in studies where retinal oxygenation is measured during systemic hyperoxia (Hardarson et al., 2006; Palkovits et al., 2014). However, no such relationship was found in our study, possibly due to the narrow range of systemic oxygenation from pulse oximeter readings, but 9 out of 10 participants were within 95-98% in systemic oxygen saturation. This narrow range of only 4% variation in oxygen saturation is comparable to the standard deviation of the retinal oximetry measurements.

Smoking did not have any effect on retinal oxygen saturation in our study. However, this must be interpreted with caution, as there were particular limitations to that analysis. First of all, only current smokers were included in the smoking group and they were less than ten percent of the total study population. Secondly, the entire smoking history was not evaluated; partly because of the low number of smokers in the population but mainly as our primary interests were the possible effects of carboxyhaemoglobin on the results. Carboxyhaemoglobin may be elevated in current smokers and can interfere with retinal vessel oxygen saturation measurements due to its very similar colour to oxygenated haemoglobin. In the study by Jani et al. (2014) on normative data on retinal vessel oxygenation in healthy individuals, current smokers did not show any differences in retinal vessel oxygen saturation. The effects of smoking on retinal vessel oxygenation need, however, further evaluations in a paired study of groups of non-smokers and smokers.

Participants with known systemic hypertension were not excluded from the study if their blood pressure was under good control on antihypertensive therapy.

Systemic medications have been suspected to affect retinal vessel diameter but no evidence of significant effect on retinal vessel diameter was found evaluating common medications in the Beaver Dam Eye Study (Wong et al., 2005). Moreover, this normative database will be used as a control group for our study on patients with neovascular AMD as well as on further studies on ocular and systemic diseases. It is expected that some patients in those study groups will also have systemic hypertension that can then be controlled for.

#### *5.1.1.6 Limitations of the study on healthy persons*

The studies on retinal oximetry in healthy persons have several limitations, some have already been discussed here, such as the relatively few individuals 70 years and older as well as the lack of concomitant blood flow measurements for determination of total oxygen extraction from the retinal circulation. Other limitations are first of all the cross-sectional nature of this study, but longitudinal data would of course be more accurate in determining the effects of aging on retinal oxygenation. However, longitudinal studies are extremely expensive and difficult in practice and therefore, the cross-sectional design was chosen here.

We have not done a specific intra- and intergrader reliability measurements or intervisit repeatability measurements in this thesis. We did, however, perform intravisit repeatability measurements (see chapters 4.1.1.3 and 5.1.1.2) as well as reliability measurements by comparing right and left eyes (see chapters 4.1.1.2 and 5.1.1), although we did not calculate the intraclass correlation coefficient. Now Yip et al. (2014) have done more thorough repeatability and reliability analyses on the Oxymap T1 oximeter, using a newer version of the software, which confirmed that the oximeter yields highly repeatable and reliable data.

The presence of cataract causes more scattering of the light and can therefore affect the measured oxygen saturation (Hardarson et al., 2014; Patel et al., 2013), as discussed thoroughly in chapter 5.1.1.4. It is well known that cataract increases successively with increasing age, but the extent and rate of progression varies tremendously between persons. The objective grading of cataract was not determined in this project and therefore could not be included in the multivariate analyses as a possible confounding factor for the measurements of the oxygen saturation values, especially in the older population. Moreover, a few of the oldest participants had already been operated for their cataract, therefore having an intraocular lens, but the effects of intraocular lenses on retinal vessel oxygen saturation measurements are also yet to be determined.

Vitreoretinal adhesion may decrease the transvitreal delivery of oxygen and thereby affect the measurements of retinal vessel oxygen saturation. The presence of vitreoretinal adhesion can often be difficult to detect clinically as well as with the Stratus OCT available in the clinic at the time of the study.

Therefore, determination of the vitreoretinal interface was not included in the study. This could also be a confounding factor in the analyses of retinal oxygen saturation and further studies are needed to evaluate the effects of vitreoretinal adhesion and posterior vitreous detachment.

### 5.1.2 Oximetry in neovascular AMD patients (Paper III)

The results of our study on retinal vessel oxygen saturation in patients with neovascular AMD suggest that the retinal oxygen metabolism may be abnormal in these patients. In the simple linear regression in the present study, the venular oxygen saturation was found to significantly increase with age in AMD patients, whereas the venular oxygen saturation significantly decreased with age in healthy persons as previously discussed. Moreover, according to the multiple linear regression, venular oxygen saturation was higher in neovascular AMD patients than healthy persons after the age of 76 years and the difference between the groups increased with age in persons older than 76 years. There is no clear explanation for this higher venular oxygen saturation in neovascular AMD patients and these results must be interpreted with caution.

Although the venular oxygen saturation was higher, it does not preclude retinal hypoxia in these patients as the retinal tissue might simply be incapable of extracting adequate amounts of oxygen from the retinal vessels, resulting in more oxygen remaining in the retinal vessels and, therefore, higher oxygen saturation values in the venules. In agreement with this, the study showed that the arteriovenous difference in oxygen saturation in persons 70 years and older was less in the AMD patients than in the healthy individuals, indicating that proportionally less oxygen was being extracted from the retinal vessels in AMD patients (assuming retinal blood flow is not increased). Retinal blood flow studies are still limited, inconsistent and inconclusive. It must nevertheless, be considered unlikely that retinal blood flow is increased in neovascular AMD.

Several reports have confirmed choroidal ischaemia in neovascular AMD (Berenberg et al., 2012; Boltz et al., 2010; Goldberg et al., 1998; Grunwald et al., 1998, 2005; Metelitsina et al., 2008; Pauleikhoff et al., 1990). The diffusion from choroidal circulation might also be compromised due to increased diffusion distances from drusen, thickened Bruch's membrane and sub- and intraretinal fluid. Moreover, the increased incidence of vitreoretinal adhesion in neovascular AMD patients in comparison to healthy persons and patients with early AMD (Krebs et al., 2007; Lee et al., 2009) is believed to slow down the diffusion between the retina and the vitreous as well as the oxygen transport within the vitreous cavity. This could result in less oxygen diffusing from the retina and, therefore, less oxygen extracted from the retinal vasculature, which could lead to higher oxygen saturation in venules. On the other hand,

retinal atrophy as well as the increased number of breaks in the Bruch's membrane and subretinal neovascular membranes seen in patients with neovascular AMD compared with non-neovascular AMD and age-matched controls (Spraul et al., 1999), could possibly result in greater oxygen diffusion from the choroidal circulation to the retina. This would require less oxygen from the retinal circulation to supply the inner retina and leave the retinal venules with higher oxygen saturation. For more extensive overview on metabolic physiology in AMD see our review by Stefánsson et al. (2011).

The proposed reduced oxygen extraction in AMD patients, reflected in the lower arteriovenous difference seen in our study, would result in less oxygen going into the tissue from the retinal circulation, which might be consistent with hypoxia. However, higher venular oxygen saturation and less oxygen extraction might also be a consequence of less oxygen demand by the retinal tissue, perhaps following degeneration or decreased function in the retina. Patients with diabetic retinopathy have been shown to have hypoxic retina (Holekamp et al., 2006). Nevertheless, our study group (Hardarson and Stefánsson, 2012a) and others (Hammer et al., 2009a) have reported higher oxygen saturation in retinal venules in patients with diabetic retinopathy, in comparison to healthy persons. The same could be true in neovascular AMD, although the pathological causes might be different from those in diabetic retinopathy. In diabetic retinopathy, capillary non-perfusion and shunting is believed to be one explanation for the decreased oxygen delivery to tissue and increased venular oxygen saturation, while another is the thickening of the capillary vessel walls (Hammer et al., 2009a; Hardarson and Stefánsson, 2012a). Analysis of six eyes from three AMD patients revealed considerably thicker basal membranes of the retinal capillaries in comparison with normal eyes from individuals of similar age (Ramírez et al., 2001). If retinal capillaries in AMD patients truly have thicker basal membranes, then oxygen diffusion from the vessels to the retinal tissue can be expected to be impaired, leaving the venules with more oxygen. The same study also reported a larger proportion of non-functional capillaries in AMD patients, which resulted in no diffusion of oxygen in relevant regions, again similar to diabetic retinopathy. Moreover, the basal membrane of the inner limiting membrane was also found to be thicker in the AMD patients, which suggests decrease in the interchange of oxygen with the vitreous (Ramírez et al., 2001). Surely, due to the limited amount of study material, these results must be interpreted with caution and will need to be confirmed in a larger study. Furthermore, even if the increase in diffusion distances by thickening of basal membranes is indeed real, it may not be clinically significant.

Unlike in patients with diabetic retinopathy, retinal neovascularisation (inner retina) is generally not found in AMD, and that fact may also be viewed as evidence against inner retinal hypoxia. We must also keep in mind that the retinal oximeter measures the retinal blood vessels in the inner retina,

whereas the main pathological changes in neovascular AMD take place in the outer retina and retinal pigment epithelium. The outer retina receives oxygen from the choroid and we do not measure this directly. Although, no direct evidence can be found for inner retinal hypoxia, it is likely that the outer retina is hypoxic in neovascular AMD patients. In a study by Sheridan et al. (2009), hypoxia-inducible factors, HIF-1α or HIF-2α, were found in the choroidal neovascular membranes of all the AMD patients, who had a predominantly cellular histology of the membranes and not a disciform scar. Moreover, the HIF's were localised to the retinal pigment epithelial cells in all cases, indicating retinal hypoxia in the outer retina as HIF's are activated in the presence of hypoxia and degraded in the presence of oxygen.

Retinal vessel oxygen saturation in patients with AMD has not been measured with comparable dual-wavelength oximeter before. Previously, Schweitzer et al. (2005) used different spectroscopic techniques to evaluate reflectance spectra in AMD patients and found some abnormality in retinal oxygen saturation of AMD patients. Their study showed that arteriovenous difference was significantly reduced in patients with early AMD when compared to healthy controls as well as in comparison to patients with late AMD and the first generation descendants. However, they did not find any significant difference in oxygen saturation of healthy persons compared to patients with late AMD. They concluded that in the early stages of AMD, oxygen-consuming cells in the retina are decreasing and thereby causing less oxygen extraction from the retinal vessels. Whereas, in the late stages, the retinal circulation should have adjusted back to normal oxygen consumption, and therefore concluded that the arteriovenous difference was comparable in healthy persons and patients with late AMD. Here, it must be kept in mind that their evaluation technique was considerably different from ours. Moreover, they were evaluating both forms of late AMD together and they did not analyse the effect of aging on arteriovenous difference. In our study, the arteriovenous difference was significantly different in the healthy individuals in comparison to the neovascular AMD patients, which could be explained by the opposite trends of arteriovenous difference with increased age. The arteriovenous difference increased with age in the healthy individuals, whereas it decreased with age in the patients with neovascular AMD.

#### *5.1.2.1 Limitations of the study on neovascular AMD*

Our study on retinal vessel oxygen saturation in neovascular AMD patients compared with healthy persons has several limitations. First of all, the retinal oximeter measures inner retinal blood vessels, whereas the pathological process in neovascular AMD generally originates in the outer retina and choroid.

Another limitation is that our healthy control group could not be age-matched to the patient group, as it proved to be extremely difficult to find persons 80 years and older with healthy eyes. Our study included 12 healthy persons over 66 years

of age, which was the age of the youngest AMD patient. However, the oldest AMD patient was 95 years old but none of the healthy participants exceeded 80 years of age. This subgroup of the oldest healthy individuals is, therefore, neither large enough nor adequately age-matched for acceptable subgroup analysis. In order to account for the differences in age between the two study groups, a multiple linear regression was performed. This model can, at least partially, adjust for the age factor as well as other possible confounding variables. The final multiple linear regression models for explanation of venular oxygen saturation and arteriovenous difference showed a highly significant difference in oxygen saturation between the two study groups. The models also indicated a significant difference in the effect of age, depending on whether AMD was present or not. However, these results must always be interpreted with caution, as the models are purely mathematical and can only adjust mathematically for the parameters we include as possible confounding factors. The models do not take into account the various physiological changes occurring in the body with age as well as the physiological effects of those more frequently seen in association with AMD, like atherosclerosis and hypertension, but more than half of the AMD patients had selfreported hypertension, whereas this was the case for less than one-fifth of the healthy persons.

Further possible limitation to the study might be potential dominant data points. The statistical analyses were therefore tested for all (abnormally) influential data points, revealing some instability in the results if one or two of the most influential points were omitted from the analyses. In the simple linear regressions of oxygen saturation in relation to age, the slope for venules and arteriovenous difference in the AMD patients became statistically insignificant if those influential data points were removed. However, removing influential data points did not affect the final conclusions of the multivariate analyses, although the coefficient estimates changed. The overall conclusions of the study were therefore not changed.

Another possible limitation of our study was that we only measured vessels 8 pixels in diameter or larger (8 pixels equals approximately 74 micrometres (Blondal et al., 2011)) since the oximetry measurements have been shown to be less reliable in narrower vessels. This results in the macular branch vessels not being measured, as they are generally narrower than 8 pixels. As the macular vessels may be more relevant for studying oxygen saturation in AMD patients, we plan to improve our oximetry technology to be able to carry out reliable measurements at these narrow vessels.

The oximetry studies were all cross-sectional in nature and it is impossible to determine the causative effects of the abnormal retinal oxygen metabolism in neovascular AMD patients, that is, whether it is causing the pathology in the disease or is one of the consequences of the disease.

Finally, the quality of oximetry images may possibly change with age, as discussed thoroughly in chapter 5.1.1.4, or by diseases and, thereby, affect the oxygen saturation measurements. This needs to be further explored in future studies.

In summary, several factors may have confounded the results of this study. However, the opposite trends of venular oxygen saturation with age in neovascular AMD patients in comparison to healthy persons, where it increases with age in AMD patients but decreases with age in healthy persons, are evident. Moreover, multivariate analyses suggest that after a certain age, retinal venules have higher saturation in neovascular AMD patients compared with healthy persons.

## 5.2 The Epidemiological Study on AMD (Paper IV)

This study includes data on all intravitreal ranibizumab injections performed on treatment-naïve patients with neovascular AMD in the defined population of Iceland during March 2007 throughout December 2010. Therefore, it is possible to estimate the annual incidence of neovascular AMD as well as the economic burden for the population due to neovascular AMD. This is important not only as the disease itself causes marked morbidity but also as the available treatment has increased the load for ophthalmology service and drug costs tremendously in recent years.

### 5.2.1 Annual incidence of neovascular AMD

Iceland has a relatively high number of ophthalmologists per capita and patients have fairly unrestricted access to ophthalmologists. Consequently, a high proportion of persons experiencing visual loss from neovascular AMD is expected to seek the service of an ophthalmologist and be referred to the centralized eye service to receive anti-VEGF treatment promptly if needed. However, it can also be expected that some patients may be too frail, physically and/or mentally to request medical assistance so the predicted annual incidence of neovascular AMD in this study, 0.29% in the population 60 years and older, might be considered the minimum annual incidence of neovascular AMD in the population of Iceland – although we believe it to be very close to the absolute incidence. In Germany, Neubauer et al. (2010) estimated that 50 000 persons are diagnosed with neovacular AMD each year, which would be approximatelly 0.24% of the population 60 years and older (Naegele, 2011). Comparable estimation in the United Kingdom, suggests 26 000 patients will develop neovascular AMD patients each year, or 0.20% of the population 60 years and older (Colquitt et al., 2008). Our population-based calculated annual incidence is slightly higher than the estimated annual incidence data from these populations.

In agreement with the large population-based studies on AMD, the annual incidence of neovascular AMD in our study increased with advancing age. In the age-group 60-69 years the annual incidence was found to be 0.06%, while it was 0.81% in patients 80 years and older. Similarly, the calculated annual incidence of neovascular AMD in the Blue Mountain Eye Study was for the same age-groups 0.10% and 0.72%, respectively (Mitchell et al., 2002). Additionally, the annual incidence for patients 85 years and older in our study was more than twofold in comparison to those 75-79 years and almost twentyfold compared with the youngest participants of 60-69 years of age. This is also in agreement with the recent Icelandic population-based study that found persons 85 years and older to have tenfold higher prevalence of late AMD compared to persons 70-74 years old, and fivefold higher for those 75-79 years old (Jonasson et al., 2011). In our Icelandic Genetics Study on AMD, a 37% increase of late AMD was found in persons 95 years and older compared to those 75 years and older (Geirsdottir et al., 2007).

### 5.2.2 Number of injections and economic impact

The debate on the optimal dosing scheme for anti-VEGF therapy in neovascular AMD is still ongoing. The monthly-dosing regimen of the MARINA (Rosenfeld et al., 2006) and ANCHOR (Brown et al., 2006) studies that was shown to be effective in improving the visual acuity of patients with neovascular AMD, is difficult to accomplish in regular clinical settings. Therefore, several studies were designed to evaluate the efficacy of less intense treatment regimens in order to decrease the burden on patients and ophthalmology services. Less than a year after the publication of the MARINA and ANCHOR trials, the PrONTO study was the first to present preliminary 12-months results of variable-dosing regimen in 40 patients with neovascular AMD in the US. After the initial loading dose of three monthly injections, the patients were treated on as-needed scheme (pro re nata) guided by OCT findings. The patients received a mean of 5.6±2.3 injections (range 3 to 13) over a period of 12 months and visual acuity improvements were considered comparable to the results of the monthly-dosing regimens in the larger population-based studies (Fung et al., 2007).

When the ranibizumab treatment began in Iceland in March 2007, patients in our clinic received treatment on an as-needed basis after the initial loading dose of three monthly injections. If a retreatment was needed, that generally included a set of three additional monthly injections, but otherwise follow-up visits were subsequently extended by 2 weeks till signs of active disease were observed. This regimen resulted in a mean number of 5.0±2.3 injections for the first 12 months of participation in our study, which was comparable to the results of the PrONTO Study described above (Fung et al., 2007). It was also in agreement with a prospective study on 138 patients with neovascular AMD in Switzerland, in which the as-needed regimen with OCT guidance was

followed from baseline (Rothenbuehler et al., 2009). Their patients received a mean of 5.6±2.9 injections (range 3 to 11) over 12 months and comparable results of visual acuity measurements. A retrospective study from Denmark, in which patients with neovascular AMD who initiated ranibizumab treatment during 2007 were included (n=555), showed that patients received on average 5.2 injections over the first year of study but all patients were treated with initial 3 monthly injections followed by a variable-dosing regimen according to signs of choroidal neovascularisation activity (Lindekleiv and Erke, 2013; Rasmussen et al., 2013).

Due to the confined Icelandic population and the centralised anti-VEGF therapy in our clinic, it is possible to assess the service load on patients and ophthalamologists as well as the costs involved in offering ranibizumab treatment to patients with neovascular AMD in a Caucasian population. During the study period, approximately 1200 intravitreal ranibizumab injections were given per year for neovascular AMD in Iceland. However, if each patient had been included in a fixed monthly regimen, the number would have been close to 2900 ranibizumab injections per year and for our clinic that would have been as good as impossible, without major compromises in other areas.

As population projections anticipate substantial growth of the older age-groups in the next decades, the burden of AMD might become even greater (Lindekleiv and Erke, 2013), although predictions are particularly difficult with the large number of uncertainty factors in relation to risk factors, pathogenesis and nature of AMD. As studies have now shown that the monthly-dosing regimen is significantly more effective in regard to final visual acuity (CATT Research Group, 2012), it is possible that ophthalmology clinics will have to adjust to that, as it appears that anti-VEGF drugs will continue as the main treatment for neovascular AMD for the foreseeable future. The high cost of ranibizumab, will however, be unaffordable for many government funded clinics (as was the case in our clinic), and it may be expected that more and more will change to the much cheaper bevacizumab, which still has not been shown to be inferior in comparison to ranibizumab (Chakravarthy et al., 2013) or to the most recently approved treatment for neovascular AMD, aflibercept. Aflibercept must be considered advantageous in terms of future treatment of AMD as studies have shown that it can be injected bimonthly after the initial three monthly injections with same positive effects on visual acuity (Heier et al., 2012). This will decrease the burden of treatment for both patients and ophthalmological services with the potential cost savings and lower cumulative risk of injection-related adverse effects.

### 5.2.3 Limitations of the AMD incidence study

There are some limitations to our study that should be considered. First of all, data on visual acuity for patients at 12 months to evaluate the efficacy of our

treatment regimen were unfortunately not accessible. The reason is that our clinic did not have the capacity to take care of all the follow-up visits so the majority of patients continued their follow-ups in the private clinics, and patients were only referred to the University Eye Clinic for re-treatment if needed. Secondly, our study is based on pragmatic clinical practice and the ophthalmologists were given guidelines to the retreatment decision-making. However, the guidelines were not prescriptive and the eventual decision to retreat and rescreen patients was based on the consultant's conclusion. Therefore, decisions were not uniform between various consultants and different patients.

# 6 Conclusions

This thesis demonstrates a significant decrease in venular oxygenation in healthy individuals with increasing age, while the reverse is true for treatment-naïve neovascular AMD patients, where venular oxygen saturation appears to increase with increasing age. This is the first study on retinal vessel oximetry in patients with neovascular AMD and it suggests that retinal oxygen metabolism may indeed be abnormal in these patients. Further studies are required to confirm these findings as well as to determine the retinal vessel oxygen saturation in other stages of AMD. More challenging will be to construct a prospective longitudinal oximetry study in order to establish the causative relationship of the abnormal retinal oxygen metabolism in AMD.

The thesis presents a standardised protocol for acquisition and analysis of images from the new version of the retinal oximeter, Oxymap T1, and provides the first complete normative database for retinal oximetry in a healthy Caucasian population. In the beginning of this project, retinal oximetry had been shown to be reliable and repeatable with previous versions of the Oxymap oximeter as well as different types of oximeters. The present study confirms the reliability and repeatability for this new version of retinal oximeter. Validation of the new oximeter and detection of all its potential technical limitations are important for future improvements and implementation of this technique in clinical practice. The ultimate aim is that retinal oximetry will be of help in diagnosing and monitoring diseases as well as evaluating effects of treatments.

The project provides an estimation of the predicted annual incidence of neovascular AMD in Iceland, which is comparable to previous studies in Iceland as well as in other Caucasian populations. The treatment load with intravitreal anti-VEGF injections is also assessed. Such assessments are valuable when planning treatment programs and estimating costs of treatment for neovascular AMD in Iceland and for comparable Caucasian populations. Longitudinal studies evaluating the longterm effects on visual acuity of the current treatment program in Iceland are needed.

AMD is the leading cause of severe visual impairment among older persons in the Western World. With the growing elderly populations in many countries, more people are at risk of developing the disease and thus, knowledge and understanding of the disease is extremely important. AMD has been shown to have multifactorial etiology, involving complex interaction of genetic, inflammatory, oxidative and degenerative components and the results of this thesis support the role of abnormal oxygen metabolism in the pathophysiology of AMD.

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