Choroidal and retinal oximetry

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HÁSKÓLI ÍSLANDS

Súrefnismælingar í æðahimnu og sjónhimnu

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Ritgerð til meistaragráðu í líf- og læknavísindum

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Ágrip

Tilgangur

Æðar sjónhimnunnar má auðveldlega mynda gegnum sjáaldur augnanna með sérhæfðum augnbotnamyndavélum og með litrófsmælingu er hægt að mæla súrefnismettunina í æðunum sjálfum. Síðastliðinn áratug hefur hópur sérfræðinga á þessu sviði á Íslandi þróað tækni og hugbúnað sem mælt getur súrefnimettun í æðum sjónhimnunnar í mönnum án inngrips. Margar rannsóknir hafa verið gerðar sem sýnt hafa fram á nákvæmni og áreiðanleika þeirra mælinga en einnig hefur vitneskja um súrefnisbúskap sjónhimnunnar í hinum ýmsu augnsjúkdómum aukist. Þessar mælingar eru takmarkaðar við sjónhimnuæðar, en sýna ekki æðahimnu, sem sér ytri hluta sjónhimnu fyrir súrefni þ.e. ljósnemunum sjálfum. Eins eru augnbotnamyndirnar takmarkaðar við 50 gráðu sjónarhorn, meðan laser skanna augnbotnamyndavélar geta séð nær allan augnbotninn, allt að 200 gráðum.

Tilgangur þessa verkefnis var að þróa tækni til að bæta úr þessum tveimur takmörkunum. Í fyrsta lagi að kanna hvort hægt væri að mæla súrefnismettun í <u>æðum æðahimnu</u> með augnbotna súrefnismettunarmæli og í öðru lagi að kanna hvort hægt væri að mæla súrefnismettun í æðum sjónhimnunnar með <u>laser skanna augnbotnamyndavél</u> (e. scanning laser ophthalmoscope, SLO).

Aðferðir

Tvennskonar súrefnismælar voru notaðar til þess að mæla súrefnismettun í æðum augnbotnsins. Annar er byggður á venjulega augnbotnamyndavél (Oxymap T1, Oxymap ehf., Reykjavík) sem notar síað hvítt ljós/flass við myndatöku, hin er laser skanna augnbotnamyndavél sem notar laser ljós við myndatöku (Optomap 200Tx, Optos plc., Dunfermline, Skotland). Sami hugbúnaðurinn, Oxymap Analyzer, var síðan notaður til þess að vinna myndirnar og reikna út súrefnismettun æðanna. Hugbúnaðurinn er hannaðar sérstaklega fyrir fyrri myndavélina en aðlagaður til þess að virka með þeirri síðari. Báðar myndavélarnar mynda augnbotninn með tveimur bylgjulengdum og með því að skoða gleypni ljóss í æðum augnbotnsins á tveimur bylgjulengdum er hægt að reikna út ljósþéttnihlutfall (e. optical density ratio, ODR), en sú stærð er í öfugu hlutfalli við súrefnismettun.

<u>Súrefnismælingar í æðahimnu (grein I)</u>

Mælingar voru gerðar á 16 heilbrigðum einstaklingum, ljósum yfirlitum, (40±14 ára, meðaltal±staðalfrávik). Sex af þessum 16 voru auk þess myndaðir fyrir og eftir innöndun á hreinu súrefni. ODR var mælt fyrir æðar æðahimnunnar (blanda af slag- og bláæðlingum), vortex bláæðar og slag- og bláæðlinga sjónhimnunnar. ODR var mælt í stað súrefnismettunar þar sem kvörðun fyrir súrefnismettunarmælingar í choroid er ekki aðgengilegt enn sem komið er.

<u>Súrefnismælingar með laser skanna augnbotnamyndavél (grein II)</u>

Augnbotnamyndir voru teknar af 11 heilbrigðum sjálfboðaliðum (34±9 ára, meðaltal±staðalfrávik). Tvær myndir voru teknar af hægra auga allra svo hægt væri að meta endurtekningarhæfni tækisins. Að auki voru tvær aðferðir notaðar til þess að meta næmni aðferðarinnar: **1.** innöndun á hreinu súrefni (n=2) og **2.** mælingar á sjúklingum með miðbláæðarlokun þar sem súrefnisþurrð hafði verið áður staðfest (n=4).

Niðurstöður

Súrefnismælingar í æðahimnu (grein I)

ODR var 0,10±0,10 (meðaltal±staðalfrávik, n=16) í æðum æðahimnunnar, 0,13±0,12 í vortex bláæðum (n=12), 0,22±0,04 í slagæðlingum sjónhimnunnar (n=16) og 0,50±0,09 í bláæðlingum sjónhimnunnar (n=16). ODR var marktækt lægra í æðum æðahimnu en slagæðlingum sjónhimnu (p=0,0012). Við innöndun á hreinu súrefni (n=6) lækkaði ODR um 0,035±0,028 í æðum æðahimnunnar (p=0,028, parað t-próf), 0,022±0,017 í slagæðlingum sjónhimnunnar (p=0,022, parað t-próf) og 0,246±0,067 í bláæðlingum sjónhimnunnar (p=0,0003, parað t-próf).

Súrefnismælingar með laser skanna augnbotnamyndavél (grein II)

Meðaltals súrefnismettun í slagæðlingum sjónhimnu var 92%±13% (meðaltal±staðalfrávik, n=11) og í bláæðlingum 57%±12% (p<0,0001, parað t-próf). Staðalfrávik fyrir endurteknar mælingar sömu sjónhimnuæða var 3,5% fyrir slagæðlinga og 4,4% fyrir bláæðlinga. Fyrir sjúklinga með meginbláæðarlokun (n=4) var meðaltals súrefnismettunin í bláæðlingum 23%±3% í sjúka auganu og 59%±3% í heilbrigða auganu (p=0,0009, parað t-próf).

Ályktanir

Súrefnismælinar í æðahimnu (grein I)

Hægt er að mæla ljósþéttnihlutfall (ODR) í æðum æðahimnu hjá fólki ljósu yfirlitum. Ástæða þess að ODR mælist lægra í æðahimnu en sjónhimnu getur verið vegna hærri mettunar í æðahimnu (lægra ODR=hærri mettun), sem samræmist dýratilraunum. Einnig hefur dreifing ljóssins í æðahimnu, þar sem æðar æðahimnu liggja aftar en sjónhimnuæðar, áhrif á súrefnismælinguna í æðahimnu og veldur þannig einnig lægra ODR. Súrefnismælirinn er næmur fyrir þeim breytingum sem verða við innöndun á hreinu súrefni. Marktæk lækkun var á ODR, við innöndun, fyrir allar æðar bæði í æðahimnu- og sjónhimnuæðum.

Súrefnismælingar með laser skanna augnbotnamyndavél (grein II)

Hægt er að nota laser skanna augnbotnamyndavél til þess að mæla súrefnismettun í æðum sjónhimnu. Tækið greinir mun á slag- og bláæðlingum, og er næmt fyrir breytingum sem verða á súrefnismettun s.s. við innöndun á hreinu súrefni eða súrefnissnautt ástand í kjölfar æðalokunar. Endurtekningahæfni er góð en breytileiki, bæði innan auga og milli augna er mikill. Laser skanna augnbotnamyndavélin hefur ákveðna kosti fram yfir venjulega augnbotnamyndavél og því gæti hún mögulega, eftir frekari þróun, orðið vænlegri sem súrefnismettunarmælir fyrir sjónhimnu og jafnvel æðahimnu.

Abstract

Purpose

The retinal blood vessels are easily accessible for imaging with specialised fundus cameras. If the vessels are imaged with two or more wavelengths the relative haemoglobin oxygen saturation of the vessels can be calculated with a technique which is based on decades of research. Recently, a group of researchers in Iceland have developed a non-invasive retinal oximeter and specialised software to acquire fundus images and calculate oxygen saturation in retinal vessels. The oximeter has been extensively validated and is sensitive to changes in oxygen saturation and vessel diameter and gives repeatable and reliable results. The oximeter has also given valuable results on the oxygen metabolism in various eye diseases. These measurements are, however, limited to retinal blood vessels and cannot measure the vessels of the choroid which supply oxygen to the outer retina, including the highly metabolically active photoreceptors. Another limitation of the fundus-camera-based oximetry is that the fundus images view up to 50 degrees of the retina while other retinal cameras such as a scanning laser ophthalmoscope can view up to 200 degrees of the retina.

The overall aim of this project was to develop techniques which could address these limitations. More specifically, the first aim was, to test the possibility of measuring the oxygen saturation of choroidal vessels with a fundus-camera-based retinal oximeter and secondly, to test if the oxygen saturation of the retinal vessels can be measured with a scanning laser ophthalmoscope (SLO).

Methods

Two kinds of oximeters were used to measure oxygen saturation of retinal and choroidal blood vessels. One is based on a conventional fundus camera (Oxymap T1, Oxymap ehf., Reykjavík) which uses filtered white light for image acquisition. The other is a scanning laser ophthalmoscope (Optomap 200Tx, Optos plc., Dunfermline, Scotland) which uses lasers for image acquisition. The software, Oxymap Analyzer, processes the images from both cameras and calculates relative haemoglobin oxygen saturation for vessels. The software is specially designed for Oxymap T1 but was adapted to work with Optomap. Both fundus cameras use two wavelengths to image the fundus. The ratio between light absorbance of blood vessels at two wavelengths, optical density ratio (ODR) is inversely related to haemoglobin oxygen saturation.

Choroidal oximetry (paper I)

Images were obtained from 16 healthy and lightly pigmented individuals (age 40±14 years, mean±SD). Six of these 16 were imaged before and after inhalation of pure oxygen. ODR was measured for choroidal vessels (both arterioles and venules), choroidal vortex veins and retinal arterioles and venules. ODR was measured instead of oxygen saturation because calibration for choroidal measurements is not available yet.

Scanning laser oximetry (paper II)

Fundus images were acquired of 11 healthy individuals (age 34±9, mean±SD). Two images were acquired of the right eye of each to test the repeatability of the measurement. Two different methods

were used to investigate the sensitivity: **1.** pure oxygen inhalation (n=2) and **2.** measurements of patients with central retinal vein occlusion (CRVO), where hypoxia had been confirmed (n=4).

Results

Choroidal oximetry (paper I)

ODR was 0.10±0.10 (mean±SD, n=16) for choroidal vessels, 0.13±0.12 for vortex veins (n=12), 0.22±0.04 for retinal arterioles (n=16), and 0.50±0.09 for retinal venules (n=16). ODR is significantly lower in choroidal vessels than in retinal arterioles (p=0.0012). Inhalation of pure oxygen (n=6) lowered ODR levels in all vessel types; the decrease was 0.035±0.028 in choroidal vessels (p=0.029, paired t-test), 0.022±0.017 in retinal arterioles (p=0.022, paired t-test), and 0.246±0.067 in retinal venules (p=0.0003, paired t-test).

Scanning laser oximetry (paper II)

Mean oxygen saturation in healthy individuals was measured as 92%±13% (mean±SD, n=11) for arterioles and 57%±12% for venules (p=0.0001, paired t-test). Standard deviation for repeated measurements of the same eye was 3.5% for arterioles and 4.4% for venules. In patients with central retinal vein occlusion (CRVO) or hemivein occlusion (n=4), the mean venular oxygen saturation was 23%±3% in affected eyes and 59%±3% in healthy fellow eyes (p=0.0009, paired t-test).

Conclusions

Choroidal oximetry (paper I)

ODR can be measured non-invasively in the choroidal vessels of lightly pigmented individuals. Lower ODR of choroidal vessels than retinal arterioles may suggest higher oxygen saturation (lower ODR=higher saturation), which is in agreement with animal studies, but is also compatible with the reduced contrast of choroidal vessels at both wavelengths that is expected from scattering of light within the choroid. The decrease in ODR during pure oxygen inhalation was significant for all vessel types, which confirms that the oximeter is sensitive to changes in oxygen saturation in both choroidal and retinal vessels.

Scanning laser oximetry (paper II)

It is technically possible to measure retinal oxygen saturation with an SLO. The system detects differences between arterioles and venules and is sensitive to changes due to pure oxygen inhalation and confirmed venular hypoxia. Repeatability of the measurement is good but variability between vessels of the same eye and between eyes remains a challenge. SLO imaging has advantages over conventional fundus camera optics and with further development; SLO oximetry may provide the optimal approach to retinal and possibly choroidal oximetry.

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Abbreviations

AMD – Age-related macular degeneration

CBF – Choroidal blood flow

CCD - Charge coupled device

CRVO – Central retinal vein occlusion

CSR – Central serous chorioretinopathy

eq. - Equation

Hb – Deoxyhaemoglobin

HbO_2 – Oxyhaemoglobin

I – Light intensity from points on vessels

I_0 – Light intensity from points to the side of vessels

L/min – Litres per minute

ms – Milliseconds

nm – Nanometre

no. – Number

OD – Optical density

ODR – Optical density ratio

OCT – Optical coherence tomography

PDR – Proliferative diabetic retinopathy

pO_2 – Oxygen tension

RPE – Retinal pigment epithelium

$SatO_2$ – Haemoglobin oxygen saturation

SD – Standard deviation

SLO – Scanning laser ophthalmoscope

Ws – Watt·seconds

µm – Micrometre

1 Introduction

1.1 The human eye: anatomy and physiology

The wall of the eyeball is divided into three layers, the fibrous tunic, the vascular tunic and the neural tunic or the retina. The outermost layer of the eyeball is the fibrous tunic and it consists of the cornea, sclera and the conjunctiva. The middle layer, the vascular tunic or uvea, is composed of the choroid, ciliary body and iris. The innermost layer of the eyeball is the retina which is divided into pigmented layer and neural layer (1). The neural layer consists of different types of neural cells; ganglion, amacrine, horizontal, bipolar and photoreceptors (Figs. 1 and 2). The vitreous humour is a jelly-like fluid filling most of the inner volume of the eyeball, behind lens and to the retina.

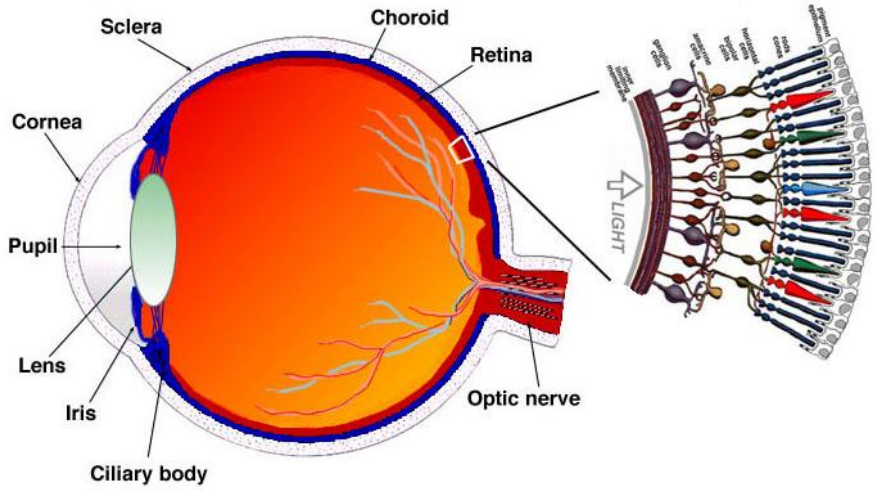

Figure 1. The human eyeball.

The wall of the eyeball consists of three layers; fibrous tunic, vascular tunic and the retina (enlarged cross-sectional diagram of the retina and choroid in figure 2). Vitreous humour fills up the inner volume of the eyeball, behind lens and to the retina. Retinal vessels can be viewed directly through the pupil of the eye. Cornea, lens and vitreous humour are almost entirely transparent to visible light. Figure from © 2013 Webvision: Attribution, Noncommercial, No Derivative Works Creative Commons license. [image on the internet] 2013, Nov 8. Available from: www.webvision.med.utah.edu/imageswv/Sagschem.jpeg

The eye has two separate vascular systems: retinal and choroidal. The retina is dependent on both vascular systems. The inner part of the retina, including the neural cells (ganglion, amacrine, bipolar and horizontal) is served by the retinal blood vessels with oxygen and nutrition. The outer retina, including the highly metabolically active photoreceptors, rods and cones, is served mainly from choroid (2) (Fig. 2).

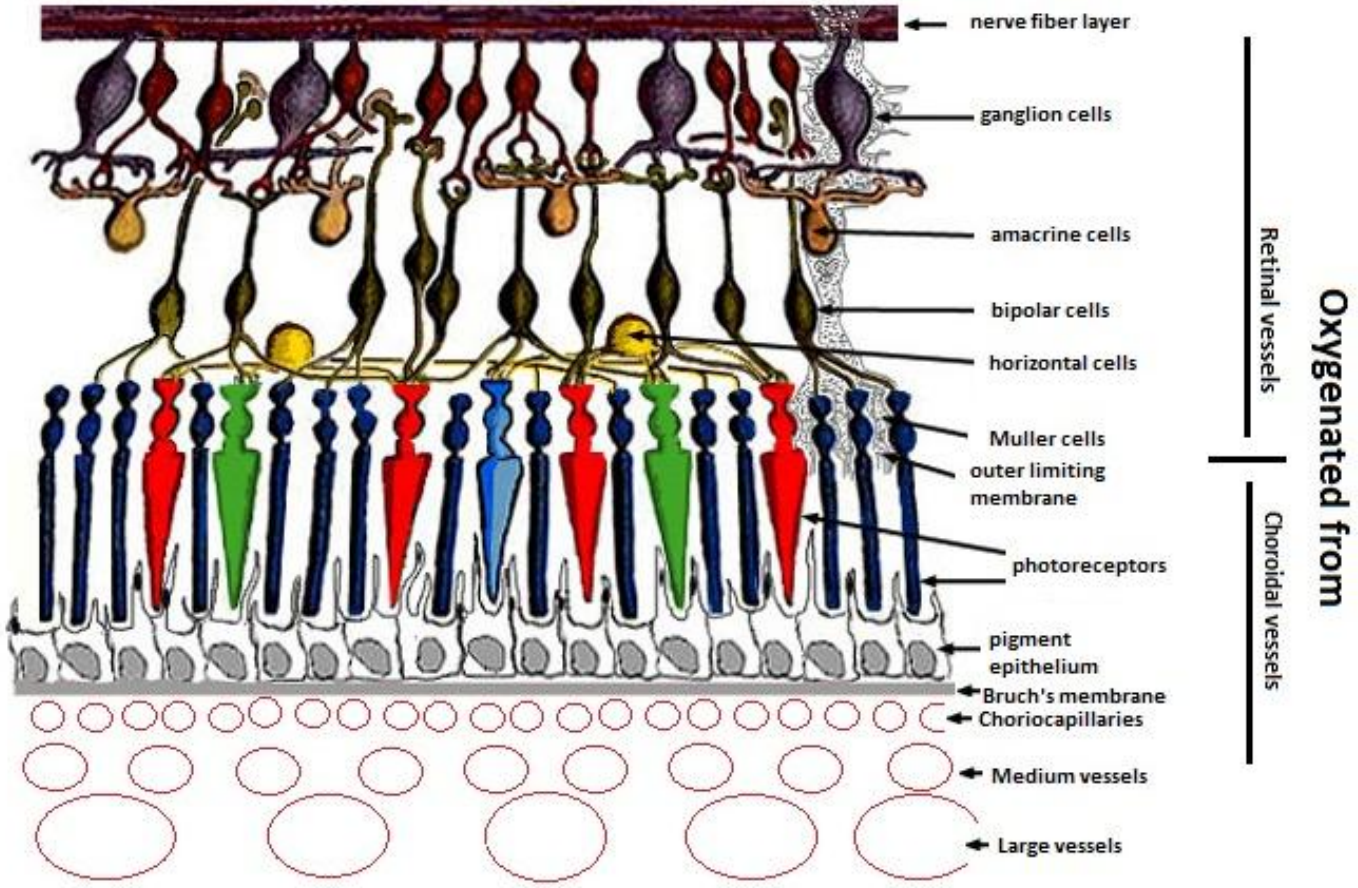

Figure 2. Cross-section of the retina and choroid and oxygenation.

Figure shows a simple cross-sectional diagram of the retina and choroid. The inner retina consists of ganglion cells, amacrine cells, bipolar cells, horizontal cells. The outer retina includes the photoreceptors and retinal pigment epithelium. The choroid consists of three layers, the choriocapillaris (next to Bruch's membrane), medium vessels (Sattler's layer) and large vessels (Hattler's layer). The inner retina is served with oxygen from the retinal vessels; the outer retina is served from choroidal vessels. Figure has been altered but original image was from: © 2013 Webvision: Attribution, Noncommercial, No Derivative Works Creative Commons license. [image on the internet] 2013, Nov 8. Available from: www.webvision.med.utah.edu/imageswv/schem.jpeg

The retinal vessels are the only blood vessels in the human body which can be viewed directly, i.e. through the pupil of the eye. Light is transmitted through the transparent optical media of the eye to the photoreceptors, the beginning of the visual pathway. Cornea, lens and vitreous humour are almost entirely transparent to visible light. The retinal layers are also almost completely transparent except for the retinal vessels, macular pigment, photoreceptors and the retinal pigment epithelium (RPE). The retinal vessels are very well distributed all over the inner retina, as if to disturb as little as possible the

incident light rays. The fovea, the area of highest visual acuity, is completely free of retinal vessels and is nourished solely by the choroid (2).

The RPE is composed of melanin containing epithelial cells and is located between the retina and choroid. The RPE and intervascular melanin in the choroid absorb light rays to prevent reflection and scattering of light within the eyeball (1). The amount of melanin or pigmentation in the RPE is correlated with iris and skin colour (3). Darker individuals have more pigment in the fundus while blonde and blue eyed individuals have much less pigment; myopic eyes also tend to have less dense pigment (4). Choroidal vessels can therefore be seen with an ophthalmoscope or on fundus images in lightly pigmented individuals.

The choroid lines most of the internal surface of the posterior sclera. Bruch's membrane is the innermost layer of the choroid and then there are three layers, the choriocapillaris (next to Bruch's membrane), medium vessels (Sattler's layer) and large vessels (Hattler's layer), which is the outermost layer next to the sclera (5).

The choroid has extraordinarily high tissue oxygen tension. The high oxygen tension in the choroid is important to allow the diffusion of oxygen from the choroid into the outer retina. Also, the great blood flow may protect the retina from thermal changes (6).

Arteriovenous difference in oxygen content in the choroid is very low. Experiments on cats have demonstrated arteriovenous difference in oxygen content of only 3% (7), and similar values have been reported for dogs and pigs, 3% to 4% (2), while the arteriovenous difference is around 35% in the retinal circulation (8).

There are four to six vortex veins in the choroidal vasculature which drain venous blood from the choroid into superior and inferior orbital veins (9).

1.2 Retinal oximetry

Oximetry is a method for measuring the oxygen saturation of haemoglobin in blood and an oximeter is the device used. The most commonly used oximeters are pulse oximeters, which measure the oxygen saturation of pulsing arterial blood, usually in a finger or an earlobe. Another kind of oximeter is a retinal oximeter, which measures oxygen saturation of retinal vessels. Both of these oximeters work in similar ways, through spectrophotometry, the basic purpose is the same, to get information on blood oxygenation from blood vessels.

Measuring oxygenation of blood helps assess the circulatory and respiratory condition of patients. It is an important evaluation in many medical conditions and for organs such as the brain, heart and lungs. The retina is highly metabolically active tissue and information on retinal and choroidal oxygenation gives vital clinical information on the metabolic state of the retina, which may improve the understanding of retinal metabolism in health and disease. Malfunction of the retinal vasculature can result in serious eye diseases, which can affect vision considerably e.g. central retinal arterial occlusion or central retinal vein occlusion. Studies on retinal oxygenation have discovered some abnormalities in retinal oxygen metabolism in the major eye diseases responsible for majority of blindness in the developed world, such as glaucoma (10, 11), diabetic retinopathy (12-14) and age-

related macular degeneration (15, 16). Their pathophysiology is still unclear and retinal oximetry could give valuable information on these presumably ischemic diseases to further the understanding of their pathophysiology and aid in diagnostic evaluation and management of these diseases.

A reliable non-invasive method for retinal and choroidal oxygen saturation measurements in humans is essential to increase the knowledge on oxygen metabolism of the human eye. Until recently, most of the knowledge on oxygenation of the eye has been based on invasive animal studies. Animal studies have given valuable information but there is still a need for an increased knowledge on the human retinal and choroidal vasculatures, both in health and disease. In this thesis the retinal and choroidal oxygen saturation of the human eye was measured with the use of two different types of devices, a fundus-camera-based oximeter and a scanning laser ophthalmoscope.

1.2.1 Principles of retinal oximetry

Oxygen is transported by the blood from lungs to the tissues. About 2% is dissolved in the plasma and 98% is bound to haemoglobin (Hb) as oxyhaemoglobin (HbO_2) (17). Haemoglobin oxygen saturation is the percentage of HbO_2 out of total haemoglobin content in blood and because most of oxygen in blood is bound to haemoglobin; haemoglobin oxygen saturation is a good measurement of the amount of oxygen in blood.

Haemoglobin oxygen saturation ($SatO_2$) is calculated according to following equation were [Hb] is the concentration of haemoglobin and [HbO_2] is the concentration of oxyhaemoglobin:

$$SatO_2 = \frac{[HbO_2]}{[Hb] + [HbO_2]} \cdot 100\% \qquad (eq. 1)$$

Measuring the haemoglobin oxygen saturation using non-invasive spectrophotometric oximetry is based on the fact that oxygenated and deoxygenated haemoglobin has different light absorption properties, i.e. different colour. Deoxygenated blood (less HbO_2) is dark red (bluish) and fully oxygenated blood (more HbO_2) has a brighter red colour.

To measure the haemoglobin oxygen saturation of blood, measurement must be done at two different wavelengths, one wavelength where the absorption of oxyhaemoglobin and deoxyhaemoglobin is different (non-isosbestic, i.e. sensitive to oxygen saturation) and one wavelength where the absorption is equal (isosbestic, i.e. not sensitive to oxygen) (17). Figure 3 shows the absorption of oxyhaemoglobin (HbO_2) and deoxyhaemoglobin (Hb) for wavelengths from 500 – 640 nm.

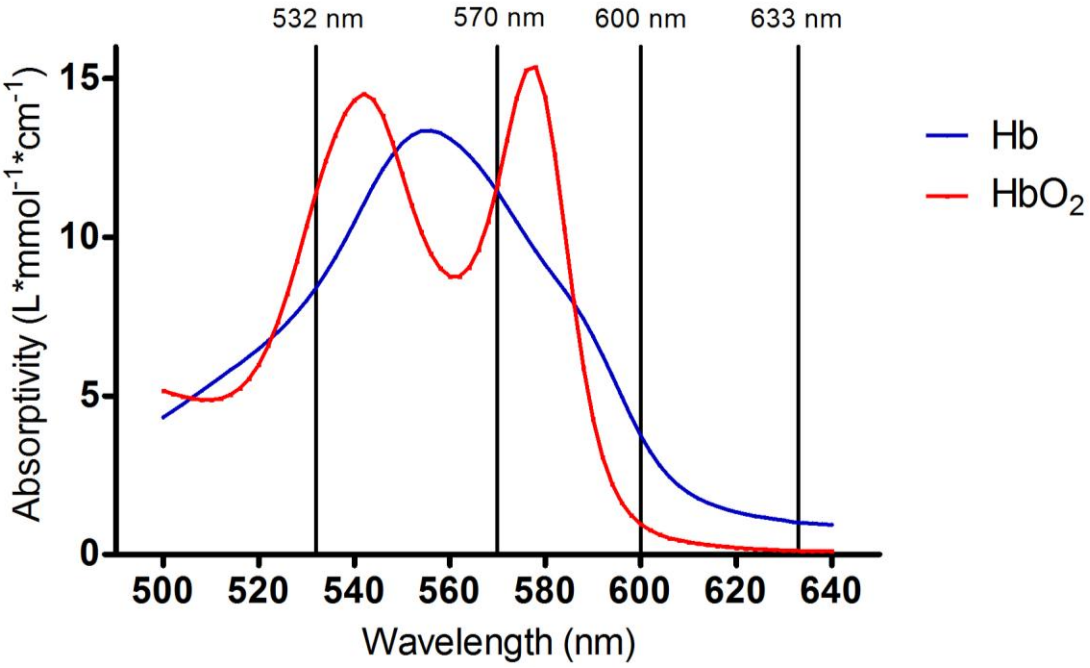

Figure 3. Light absorptivity of oxyhaemoglobin (HbO_2) and deoxyhaemoglobin (Hb).

Light absorptivity for Hb and HbO_2 is the same at isosbestic wavelength (e.g. 570 nm) but different at non-isosbestic wavelengths (e.g. 600 nm). Figure is produced with data in Zijlstra et al. (18).

Haemoglobin oxygen saturation measurements in a sample of blood are based on the Beer-Lambert's law. In regular transmission blood oximetry, it is assumed that the absorption of light is dependent on the extinction coefficient (molar absorbance) of the blood sample (***ε***), the distance through the sample (*d*) and the concentration of the sample (*c*):

$$I = I_0 \cdot 10^{-\varepsilon c d} \qquad (eq.2)$$

I = Intensity of light transmitted through the sample

I_0 = Incident light intensity (original light intensity before interaction with the sample)

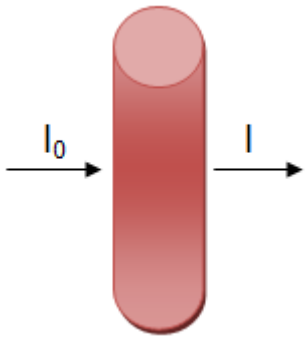

Figure 4. Light absorption of a blood sample.

I_0 is the original light intensity before some of the light is absorbed by the blood sample and I is light intensity transmitted through the blood sample.

Therefore, the light absorbance of a blood sample, at any given wavelength (λ), also called optical density (OD) is defined as:

$$OD_\lambda = log\frac{I_0}{I} = \varepsilon cd \qquad (\text{eq. } 3)$$

OD is a function of the extinction coefficient, concentration and the distance the light has to travel through the sample. At an isosbestic wavelength, where extinction coefficients for Hb and HbO_2 are identical, the OD for blood is only dependent on *c·d,* not on oxygen saturation. At a non-isosbestic (oxygen sensitive) wavelength, where extinction coefficients are not the same for Hb and HbO_2, the OD is dependent on *c·d* and oxygen saturation. By taking the ratio between two OD's at two distinct wavelengths (optical density ratio, ODR), one isosbestic and one non-isosbestic, the distance and concentration factors cancel out but not the oxygen saturation (19). ODR is therefore sensitive to oxygen saturation and has an approximately linear relationship to haemoglobin oxygen saturation (19, 20).

$$ODR = \frac{OD_{non-isosbestic}}{OD_{isosbestic}} \qquad (eq. 4)$$

$$SatO_2 = a \cdot ODR + b \qquad (eq. 5)$$

(*a and b are constants*)

Above described principles are based on the relationship between light transmittance through a solution, such as blood, where I and I_0 are measurements of light intensity before and after attenuation of oxy- or deoxyhaemoglobin in the solution (clear solution with only oxy- and deoxyhaemoglobin). For retinal oximetry some approximations must be made. It is clearly impractical to measure transmittance

of light through retinal vessels where it is not possible to measure the light behind the blood vessels in the retina. Therefore, reflected light is measured instead.

Light intensity, I and I_0 from equations 2 and 3, can be estimated based on reflected light from the fundus. I_0 is light intensity from the background to the side of the vessels, light that has not interacted with the blood in the vessels. I is the light intensity reflected from vessels, light that has partly been absorbed by the blood in the vessels (19). By selecting light intensity reflected from the background, close to the vessel, I and I_0 are affected by similar factors except for the blood inside of the vessel itself (Fig. 5). However, light reflection measurements of retinal blood vessels are much more complex than a simple transmission measurements through a solution, light reflected is not only influenced by oxy- and deoxyhaemoglobin, instead light can be scattered from e.g. blood cells, vessel walls and pigmentation, all this makes saturation calculations inaccurate (21-23).

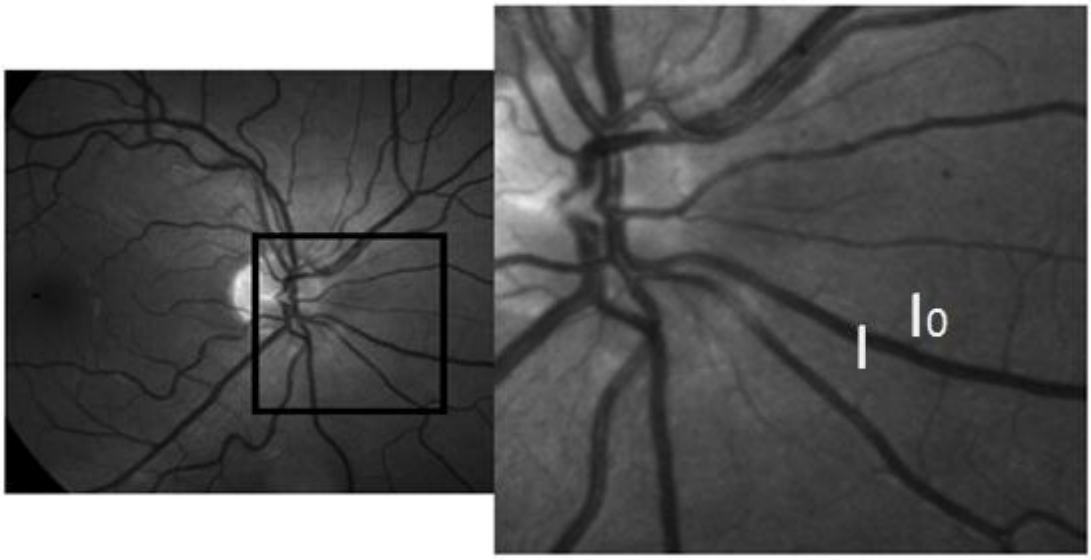

Figure 5. Retinal reflectance oximetry.

In the retina, haemoglobin oxygen saturation of blood vessels can be calculated according to Beer-Lambert's law on light absorption by measuring light reflected instead of light transmitted. I_0 is light intensity reflected from background, close to the vessels (light that has not interacted with blood in the vessel) and I is light intensity reflected from vessel.

1.2.2 Development of retinal oximetry

Non-invasive spectrophotometric retinal oximetry has been studied for over 50 years. It has evolved from film photography (20) to digital photography (24-28), photoelectric imaging (21), imaging spectroscopy (8, 29-31), scanning laser ophthalmoscopy (SLO), with (32) and without (33, 34) the use of adaptive optics, and optical coherence tomography (OCT) (35-37). All methods use in some way different imaging approaches but the theoretical foundation is the same in all cases. The retina must be imaged by two or more wavelengths and the absorption difference between oxyhaemoglobin and deoxyhaemoglobin is utilized and from that information the relative haemoglobin oxygen saturation can be calculated.

Retinal oximetry research started in 1959 with the use of film photography, when Hickam, Sieker and Frayser published the first paper on dual-wavelength non-invasive retinal oximetry (20). Using special filters, Hickam and his colleagues measured the diameter and oxygen saturation of human retinal vessels from fundus photographs. This was the first time the oxygenation of the human retina was measured noninvasively and following these studies many techniques have been developed. Laing et al. (38) continued in 1975 with a similar method, a two wavelength photographic method used on rabbits. The method demonstrated sensitivity for changes in retinal oxygen saturation. Measurements of retinal arterial saturation compared to femoral arterial oxygen saturation were in good agreement. In 1988, Delori et al. (21) followed with a paper on three-wavelength photoelectric retinal oximetry technique. The use of a three-wavelength method can compensate for effects of light scattering (22). Their retinal oximeter scanned the retinal vessels in real-time using three wavelengths and was able to measure optical density and calculate oxygen saturation of each scanned vessel segment.

Oximetry studies continued and in 1999, Schweitzer et al. (8) and Beach at al. (24) published results on non-invasive retinal oximetry. Schweitzer et al. used a fundus camera adapted spectrograph and a detector system allowing determination of oxygen saturation and vessel diameter of arterioles and venules of the retina using multiple wavelengths. This method only allowed a measurement of a small area of the fundus at a time (1.5 mm x 40 µm slit). Beach et al. and co-workers used a dual-wavelength method similar to the one Hickam et al. used forty years before but with digital imaging and computer technology. The system used for oximetry fundus imaging was a modified fundus camera coupled to an image splitter and 18-bit digital camera. The retinal vessels were imaged simultaneously at two wavelengths (569 and 600 nm), which eliminated the effect of eye movement. The fundus was imaged under different oxygen breathing mixtures and an attempt was made to correct for vessel diameter and fundus pigmentation.

Similar photographic methods have been reported by Crittin et al. (26) and Hammer et al. (27) with the use of two or more wavelengths. In 2002, Crittin et al. imaged the retina at four different wavelengths and then used two, 569 and 600 nm, to calculate haemoglobin oxygen saturation for retinal vessels. The system was fundus camera based with an image splitter, where the fundus image was split into four spectral bands and images recorded with an intensified CCD (charge coupled device) camera. Few years later, in 2008, Hammer et al. reported a similar approach. Again, a fundus camera based system with the use of a dual wavelength transmission filter, colour CCD camera and special software. Images at two different wavelengths were acquired simultaneously of the fundus (548 and 610 nm) and haemoglobin oxygen saturation of retinal vessels was calculated using the same basic principles as used by Beach et al. (24). Ramella-Roman et al. (28) also reported results on a custom built multi-aperture camera system adapted to a fundus ophthalmoscope and capable of acquiring six images in different wavelengths of the fundus simultaneously. Calculation of haemoglobin oxygen saturation was tested in vitro and in vivo and by using two different methods.

Hyperspectral imaging techniques (utilizing many wavelengths) have been used by Khoobehi et al. (29), Yoneya et al. (30), Mordant et al. (39, 40), Johnson et al. (31) and Kashani et al. (41). Yoneya et al. measured patients with central retinal vein occlusion with the use of a Fourier transform-based

spectral retinal imaging (SRI). The system produced a topographical colour map of the oxygen saturation of every pixel of a 35 degree fundus image. Information from fluorescein angiography on capillary non-perfusion and dye leakage was correlated with oxygen saturation to validate if the SRI oximetry method could quantify retinal ischemia. Khoobehi et al. measured haemoglobin oxygen saturation in monkey eyes with the use of fundus camera based hyperspectral imaging system. The size of the fundus images was also 35 degrees and the imaging took 8 seconds and is therefore dependent on non-moving eye like the imaging by Yoneya et al. where scanning took 6 seconds. Following these studies, Johnson et al. reported on a study on snapshot hyperspectral imaging where the spectral imaging takes only about 3 ms and is therefore free of motion artefacts and pixel misregistration. The computed tomography imaging spectrometer acquired a fundus image of healthy subjects in 50 spectral bands (spatial spectral image cube) but used only three wavelengths to calculate for haemoglobin oxygen saturation (three wavelength oximetry algorithm (22)). Kashani et al. used the same device as Johnson et al., a hyperspectral computed tomographic imaging, and imaged rabbit's retinas and calculated the haemoglobin oxygen saturation using 28 wavelengths. Another hyperspectral imaging technique was published by Mordant et al. who used a hyperspectral fundus camera to measure the oxygen saturation from blood samples placed in a model eye with different amount of reflectivity from the background (39). In vivo measurements on healthy individuals were also made (40). The hyperspectral fundus camera takes about 10-15 minutes to sequentially acquire all the spectral data of all wavelengths between 500 and 650 nm (2 nm intervals).

Optical coherence tomography (OCT) is an advancing imaging modality in ophthalmology. OCT uses low-coherence light and ultra-short laser pulses to produce non-invasively, cross-sectional images of the internal structures of the retina by measuring their optical reflections (42). The use of OCT for retinal oximetry is quite new and was first reported in 2007 by Kagemann et al. (35) and more recently by Ye et al. (36) and Yi et al. (43). OCT oximetry could give at same time information on the cross-sectional structure of the retina and oxygenation.

The use of a scanning laser ophthalmoscope (SLO) for retinal oximetry will be covered in later chapter especially discussing SLO oximetry (see *1.4.1.Scanning laser ophthalmoscope for oximetry*).

The overall aim of all of the oximetry techniques, described above, is the same, to measure retinal haemoglobin oxygen saturation in humans (and animals) in a fast, non-invasive and accurate way. The major drawbacks of many of the techniques is the limitation in measurements of a small area of the fundus, some are not safe or practical to use on humans yet and all of them only measure the retinal vasculature, not the choroid (measurements by Yoneya et al. were from the fundus as a whole and it was not possible to separate the influence from choroid). The retina is served to a large degree from choroid as well as the retinal vessels and it is therefore important to be able to measure oxygenation of both vasculatures. Presently, there are only two commercially available retinal oximeters, Oxymap T1 from Oxymap ehf. (Reykjavik, Iceland) (16) and one from Imedos Systems (Jena, Germany) (27). Again, both use very similar approaches, the principles for the haemoglobin oxygen calculation are same but technical details differ.

1.2.3 Oxymap T1, spectrophotometric, fundus-camera-based, retinal oximeter

Oxymap T1 and the Oxymap Analyzer software (specialized software to analyse oximetry fundus images, described in detail in *3.1.2. Oxymap Analyzer software*) were developed in Iceland (Oxymap ehf.). The principle behind calculation of haemoglobin oxygen saturation is based on work by Hickam et al. and Beach et al. (20, 24). Numerous studies have been done over the last decade by several research groups around the world and results have shown that the oximeter detects difference between retinal arterioles and venules, is sensitive to changes in oxygen saturation (25, 44) and vessel diameter (45) and gives reliable and repeatable results (46, 47). Following is a short overview of the main results from these studies.

An oxygen inhalation (hyperoxia) study was performed to test the sensitivity of the oximeter. Results showed a significant increase in oxygen saturation for both the retinal arterioles and venules when subjects inhaled pure oxygen and also a significant decrease in vessel diameter (44). The repeatability has been tested for both the oxygen saturation and vessel diameter. The standard deviation between repeated oxygen saturation measurements of the same vessel is 1.0% in arterioles and 1.4% for venules (47). For diameter, the coefficient of variation for repeated measurements is 3.5% for arterioles and 2.8% for venules (45). Low variability between measurements demonstrates the robustness of the technology.

Additionally, a large study of healthy volunteers has been performed that provides normative data on spectrophotometric retinal oximetry for Caucasian Icelanders at the age of 18-80 years (46). This study measured the effect of age, gender, ocular perfusion pressure and showed that with increased age oxygen saturation of arterioles is stable while venular oxygen saturation decreases, both for males and females.

Numerous studies on retinal oxygen metabolism have also been done with the Oxymap T1 oximeter (or previous prototypes) in several major eye diseases e.g. glaucoma (11, 48), age-related macular degeneration (16, 49), diabetic retinopathy (13), retinitis pigmentosa (50), central retinal vein occlusion (51), branch retinal vein occlusion (52). Studies have also been done on systemic diseases such as Eisenmenger´s syndrome (53).

1.3 Choroidal oximetry

Measuring the oxygenation of the choroidal vasculature non-invasively is more complicated and challenging than measuring the retina, primarily because of the anatomy; the choroidal vasculature lies posterior to the retinal vasculature, behind the light absorbing retinal pigment epithelium. However, measurements on choroidal vasculature have also been attempted along with retinal oximetry. The first study was published in 1961 when Broadfoot et al. (54) reported on a modified ophthalmoscope capable of non-invasively detecting changes in choroidal oxygen saturation in rabbits and humans. This involved photoelectric measurements of light, at three different colours, reflected from the ocular fundus during deep normal breathing and apnoea. Fundus reflectance changed with different stimulus, suggesting that the measurement was sensitive to changes in oxygen saturation. Reflectance measurements were from the fundus as a whole and therefore most likely influenced by changes in

oxygen saturation from both choroidal and retinal blood. In 1975, Laing et al. (3) reported on a non-imaging spectrophotometric choroidal oximeter that, following calibration, was able to continuously quantify choroidal oxygen saturation. It was composed of a fundus-monitoring unit, dual-wavelength (650 and 805 nm) light source and electrical system for synchronous processing of signals and calculation of oxygen saturation. Light reflection signals were measured from the macular area of the fundus and reflection signal, according to author, primarily influenced by choroidal blood. However, it is only the fovea, the central part of macula, which contains no retinal vessels. Macula, on the other hand, contains a dense retinal capillary network (2). Thus, similar to Broadfoots experiment, light reflection from the macular area must be determined by both choroidal and retinal blood oxygen saturation.

These studies by Broadfoot et al. (54) and Laing et al. (3) establish the possibility of detecting changes in choroidal oxygenation in humans using non-invasive spectrophotometric methods. But as previously mentioned, light reflectance signals were most likely influenced by changes in oxygen saturation in both choroidal and retinal blood. Oxygen saturation was measured by detecting changes in light reflected from the fundus and not from individual vessels as most of the previously described retinal oximetry studies have done. No other studies on choroidal oxygenation have been done to date on humans.

Semi-invasive studies have been performed on choroidal oxygenation in rats by Shahidi et al. (55) where pO_2 is measured separately in the choroid and retina using pO_2 phosphorescence imaging system. The imaging part is non-invasive but there is a necessary injection of a phosphorescence oxygen sensitive molecular probe which is not safe for human use.

1.3.1 Why choroidal oximetry?

The retina relies on both the choroidal and retinal vasculatures for nourishment. To understand pathophysiology of retinal diseases it is important to increase the knowledge of the physiology of the choroid in health and disease. Measuring choroidal oxygenation may allow study of pathophysiology of diseases where choroidal ischemia and other abnormalities in choroidal blood flow may play a role, such as age-related macular degeneration (AMD) (56, 57), diabetic retinopathy (58, 59) central serous chorioretinopathy (CSR) (60) and glaucoma (61).

In AMD, studies suggest that choroidal blood flow and perfusion is disturbed during the development of the disease (57). With choroidal ischemia, oxygen delivery will decrease from the choroid to the retina; the decrease will cause hypoxia and trigger neovascularization. Drusen, retinal elevation, tissue oedema and cystoid spaces are all features of AMD. All of these features may also disturb the delivery of oxygen from choroid to retina by increasing the diffusion distance from the choroid to the retina or from retinal blood vessels to the cells of the retinal tissue. (15). Overall, there is a degeneration of the retinal tissue which also decreases oxygen consumption of the tissue.

Recent study results on oxygenation in AMD with the use of Oxymap T1 oximeter suggests that oxygen saturation in retinal venules is increased in exudative AMD (16). The retinal arteriovenous difference is less in AMD patients compared to healthy subjects. This indicates that less oxygen is being extracted from the vessels and less oxygen extraction means less oxygen is delivered to the

tissue and that might be consistent with hypoxia. Lower arteriovenous difference and less extraction may also suggest less oxygen demand by the tissue as a consequence of tissue degeneration. These data suggest retinal hypoxia and other studies indicate choroidal ischemia (56, 57). In AMD patients, it would be ideal to be able to measure choroidal and retinal oxygen saturation along with choroidal and retinal blood flow to improve the understanding of the pathophysiology of the disease. The same applies for the other major eye diseases, where choroidal ischemia plays a role, such as diabetic retinopathy, central serous chorioretinopathy and glaucoma.

Results reported by Nagaoka et al. (58) suggest that choroidal blood flow (CBF) measured at the fovea region is decreased in diabetic patients with macular oedema. Further studies are needed to determine why this is the case, whether the decrease in CBF is a primary or secondary factor in macular oedema. Schocket et al. (59) also reported on a significant decrease in CBF in diabetic patients with proliferative-diabetic retinopathy (PDR) but further studies are also needed to understand the reasons for the change and when the changes occur through the development of the disease.

Clinical features of CSR are well known while the pathogenesis of the disease is still controversial. In CSR, there is a fluid detachment between the retina and retinal pigment epithelium at the central macula area, which disturbs visual acuity. Results by Kitaya et al. (60) suggest choroidal ischemia, where results show significantly lower choroidal blood flow in CSR than in fellow eyes.

Intra-ocular pressure is known to be a major risk factor in glaucoma but other factors may also be responsible for progression of the disease such as reduced ocular blood flow and poor regulation of blood flow. Results by Yin et al. (61) suggest impairment in the choroid in primary-open angle glaucoma such as reduced choroidal thickness at the posterior pole, reduction in the number of choriocapillaries and delayed choroidal filling.

Where pathogenesis for above mentioned eye diseases is not fully known and study results suggest choroidal ischemia, choroidal oximetry could be helpful for understanding these diseases.

Another advantage of measuring the choroidal blood oxygenation directly is that choroidal oximetry may allow direct measurements of oxygen saturation in central nervous system vasculature. This may be important in cardiovascular shock and severe injury, where peripheral oxygenation as measured for example with finger pulse oximeter may be unreliable, as the body favours vital organ perfusion including the eyes.

1.4 Scanning laser ophthalmoscope

Scanning laser ophthalmoscope (SLO) is a fundus imaging system, first introduced in 1987 by Robert Webb and co-workers (43). Scanning laser ophthalmoscope, as the name suggests, scans the retina with laser beams point by point, one line at a time, while regular fundus cameras capture the image in one single capture. SLO uses monochromatic lasers for imaging where all the light energy is delivered at one specific wavelength. Regular fundus cameras use white light imaging and even though some wavelengths are often filtered out before light hits the fundus, the light is usually of much broader wavelength range than the light from lasers. Also, with the use of SLO, single point of the retina is only

illuminated for less than 1μs while with regular fundus camera, the entire retina is illuminated for several milliseconds at a time. Dilated pupils are not needed for SLO imaging because the fundus is illuminated through a small illumination aperture with a large viewing aperture that collects the reflected light from the fundus. However, dilated pupils are crucial for good quality imaging with most conventional fundus cameras.

SLO has the following advantages over conventional fundus cameras. SLO produces a sharp detailed image of the fundus, without the need for dilation, even if there are considerable optical media opacities in the individual's eye. SLO offers also the possibility of wider field imaging. Some commercial devices can image up to 200 degree field of view of the retina (Fig. 6).

Eye motion can affect the SLO fundus image more than imaging with conventional fundus camera. SLO scans the retina and during scanning eye movement can occur. Regular fundus camera captures the whole image in one single capture and therefore eye movement is unlikely to affect the image (62).

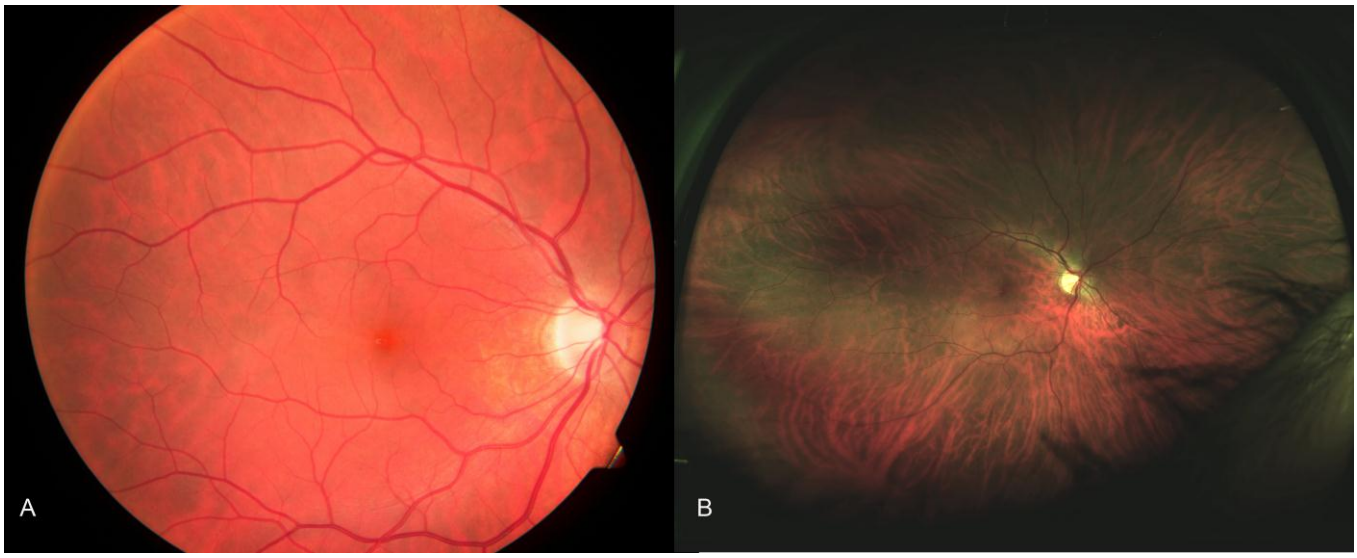

Figure 6. Comparison between regular and wide field fundus photograph.

Fundus images from the same individual. Image **A.** is acquired with a regular fundus camera and has 50 degree field of view. Image **B.** is acquired with a scanning laser ophthalmoscope (SLO) and has 200 degree field of view. SLOs offer the possibility of much wider field imaging of the fundus compared to regular fundus cameras.

1.4.1 Scanning laser ophthalmoscope for oximetry

Studies on the use of an SLO for oximetry have been published mainly by three research groups, Smith and Denninghoff et al. (34, 63, 64), Ashman et al. (33) and Li et al. (32) In 1998, Smith et al. (63) reported on a two-wavelength scanning laser Eye Oximeter (EOX) prototype, which was used for retinal oxygen saturation measurements in anesthetized swine during blood loss. Strong correlation was found between retinal arterial and femoral arterial oxygen saturation, and retinal venous saturation and blood loss. In 1999, this same research group reported on a second generation of the

EOX for studies on retinal oxygen saturation in human subjects. The EOX was mounted on a slit lamp base and four diode lasers at wavelengths 629, 678, 821 and 899 nm used to image the retina. Oxygen saturation was found to be 65% and 101-102% in the retinal veins and arteries, respectively, suggesting that the EOX is sensitive to retinal oxygen saturation (34). In 2011, Denninghoff et al. (64) reported on the use of a new modified confocal SLO (ROx-3) for blue-green oximetry in both swine and human subjects. Results on retinal oxygen saturation were in the same range as reported by other research groups. Ashman et al. (33) used a prototype SLO to measure the retinal oxygen saturation under different percentages of oxygen breathing mixtures; 10%, room air and 100%. Lasers used for imaging were 633 nm and 815 nm. A difference was seen between retinal arterioles and venules but no difference was measureable between different oxygen breathing mixtures. Li et al. (32) measured the oxygen saturation in small retinal vessels (diameter<50µm) using adaptive optics confocal SLO with two wavelengths, 680 and 796 nm. A difference between arterioles and venules was detected.

The above mentioned studies all show the possibility of oximetry imaging of the retina with the use of an SLO. SLO imaging has many advantages that might be beneficial for retinal oximetry.

2 Aims

1. Aim of the first part of the project was to develop and test a new method to measure haemoglobin oxygen saturation in the choroidal vasculature using a spectrophotometric oximeter. (Choroidal oximetry – paper I).
2. Aim of the second part of the project was to test a new device, a scanning laser ophthalmoscope, for measuring haemoglobin oxygen saturation in the retinal vasculature and to test sensitivity and repeatability of the measurement. (Scanning laser oximetry – paper II).

3 Methods

Both studies were approved by the National Bioethics Committee of Iceland and the Icelandic Data Protection Authority. All volunteers signed an informed consent before participation. Studies adhered to the tenets of the Declaration of Helsinki.

3.1 Choroidal oximetry (paper I)

Fundus images for oximetry were acquired with Oxymap T1 and ODR (inverse relationship to haemoglobin oxygen saturation) was calculated with Oxymap Analyzer (version 2.2.1/3847, Oxymap ehf., Reykjavik, Iceland).

3.1.1 Oxymap T1 retinal oximeter.

Oxymap T1 is a non-invasive, fundus-camera-based, retinal oximeter (Fig. 7). The oximeter is composed of two highly sensitive digital cameras (Insight IN1800, Diagnostic Instruments Inc., MI, USA, 1600 x 1200 square pixels), custom-made optical adapter, two narrow band-pass light filters and an image splitter. It is designed to fit as an attachment on to a Topcon TRC-50DX fundus camera (Topcon Corporation, Tokyo, Japan).

Figure 7. Oxymap T1 retinal oximeter.

Oxymap T1 is a fundus-camera-based retinal oximeter. The fundus camera is Topcon TRC-50DX and the attachment is made of two highly sensitive digital cameras, image splitter, optical adapter and light filters.

A fundus image is captured by the Topcon fundus camera, split into two by the image splitter and one image is sent to each camera sensor. One camera captures the image at 570 nm while the other captures the same area of the fundus, simultaneously, at 600 nm (Fig. 8). This is done by inserting two narrow 5 nm band pass filters (full width at half maximum transmittance) into the light path to each camera of the oximeter. Another light filter (80 nm band pass filter, 585 nm centre wavelength) is also inserted into the fundus camera itself to exclude unnecessary light exposure to subjects' eyes, only allowing light between 545 and 625 nm to exit the camera lens.

3.1.2 Oxymap Analyzer software

The composition of the oximeter and the fundus camera only works as a retinal oximeter when used with specialised software, Oxymap Analyzer, for calculation of blood vessel oxygen saturation (see *1.2.1 Principles for retinal oximetry*).

The software analyses these two spectral images (570 and 600 nm), automatically detects blood vessels, selects measurements points and measures the brightness on vessels (I) and to the side of the vessels (I_0) at each wavelength. Light absorbance of the blood vessel can be described with optical density (OD):

$$OD = log\left(\frac{I_0}{I}\right) \qquad eq.6$$

We then use equation 4 (p. 22) to calculate optical density ratio (ODR) between these two measurement wavelengths, here the non-isosbestic wavelength is 600 nm and the isosbestic is 570 nm. We therefore get:

$$ODR = \frac{OD_{600}}{OD_{570}} \qquad eq.7$$

Calibration for Oxymap T1 was done by matching ODRs from healthy individuals measured with Oxymap T1, with saturation measurements for arterioles and venules separately. The reference saturation values were taken from a study performed with a calibrated device in a separate study by Schweitzer et al. (8) According to their study, the mean oxygen saturation was 92.2% for retinal arterioles and 57.9% for retinal venules in healthy individuals. Using these values, the constants a and b were found to be: *a=-1.1755 and b=1.1917*. ODR has an inverse and approximately linear relationship to haemoglobin oxygen saturation. Haemoglobin oxygen saturation ($SatO_2$) can be calculated according to equation 5 (p. 22), after calibration.

Oxymap Analyzer software processes the two monochrome images and displays the relative haemoglobin oxygen saturation result as a pseudocolour fundus image with colours representing different oxygen saturation of the vessels (Fig. 8). Red colour represents the highest oxygen saturation or 100% and violet colour the lowest, 0% (see enlarged pseudocolour scale in Fig. 9)

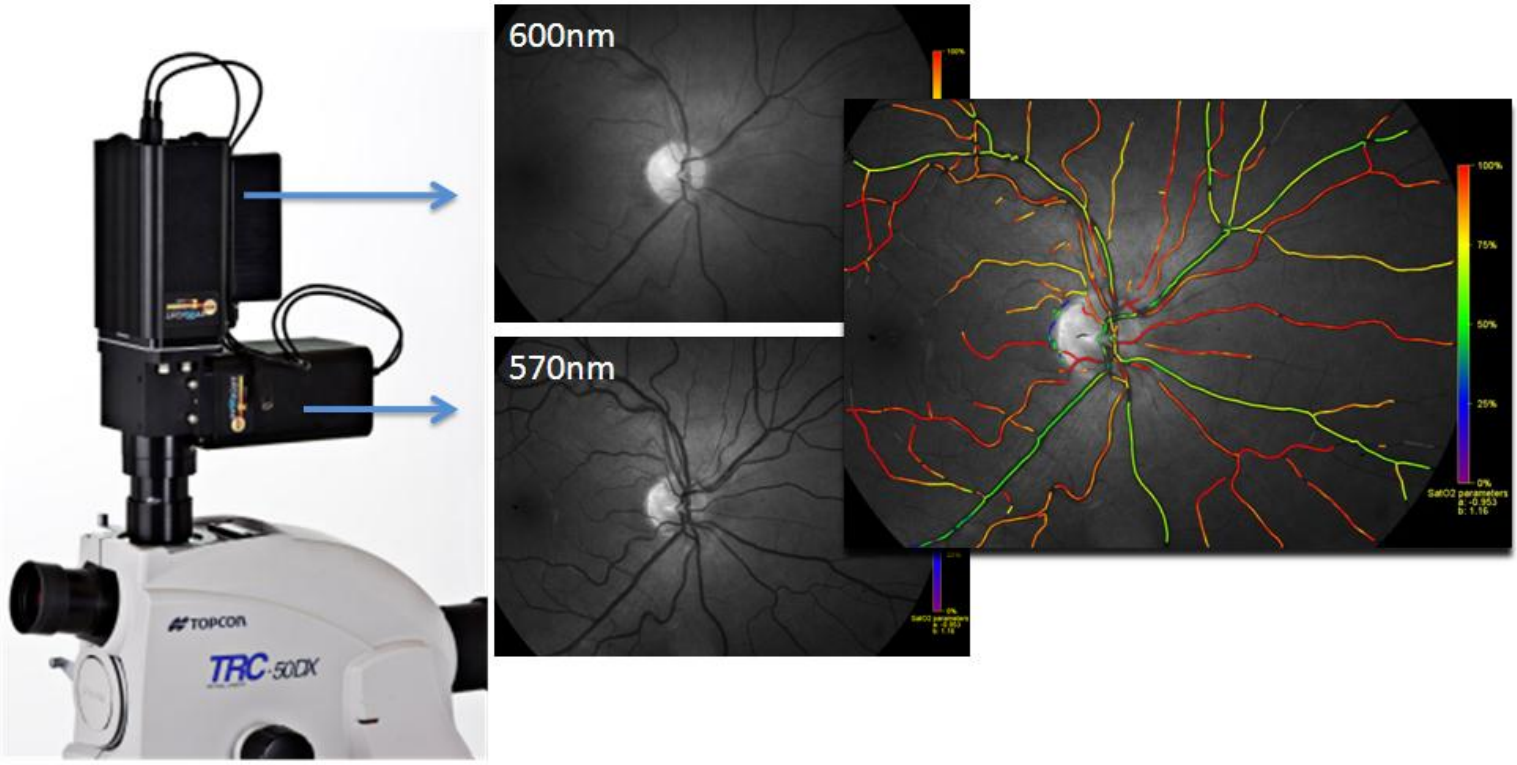

Figure 8 Oxymap T1, retinal oximetry.

Oxymap T1 acquires two monochrome images of the fundus at wavelengths 570 and 600 nm. Oxymap Analyzer software automatically processes the two spectral images and displays a pseudocolour map representing the relative haemoglobin oxygen saturation.

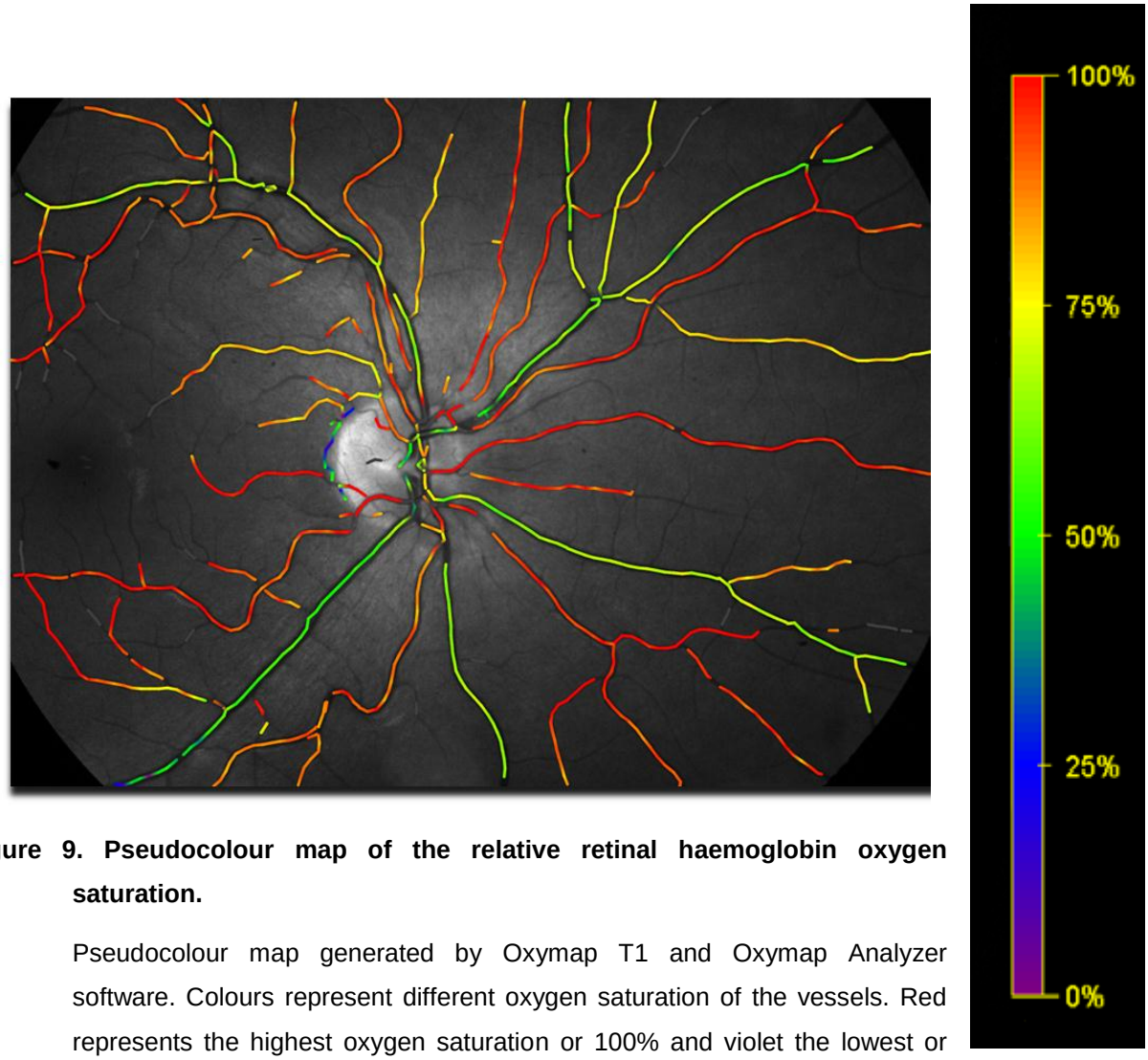

Figure 9. Pseudocolour map of the relative retinal haemoglobin oxygen saturation.

Pseudocolour map generated by Oxymap T1 and Oxymap Analyzer software. Colours represent different oxygen saturation of the vessels. Red represents the highest oxygen saturation or 100% and violet the lowest or 0%. Enlarged colour scale on right side.

3.1.3 Study protocol

For this study, sixteen subjects were selected from 148 participants from a normative study on retinal oxygen saturation in healthy individuals (46). Participants recruited were 148, excluded from analysis were 28. Exclusion criteria were bad image quality, any retinal or optic nerve disease e.g. glaucoma, age-related macular degeneration (AMD) or any other eye or systemic diseases. In all, 120 individuals were included in the study and sixteen of them were selected for the choroidal oximetry study, all were Caucasian, four males and twelve females, age of 40 ± 14 years (mean ± SD). Subjects were selected for the study solely because the choroidal vasculature was visible and measureable with the retinal oximeter (Fig. 10). All were lightly pigmented. Figure 11 shows a lightly pigmented fundus where choroidal vessels are visible and for comparison a more densely pigmented fundus, where these vessels are invisible.

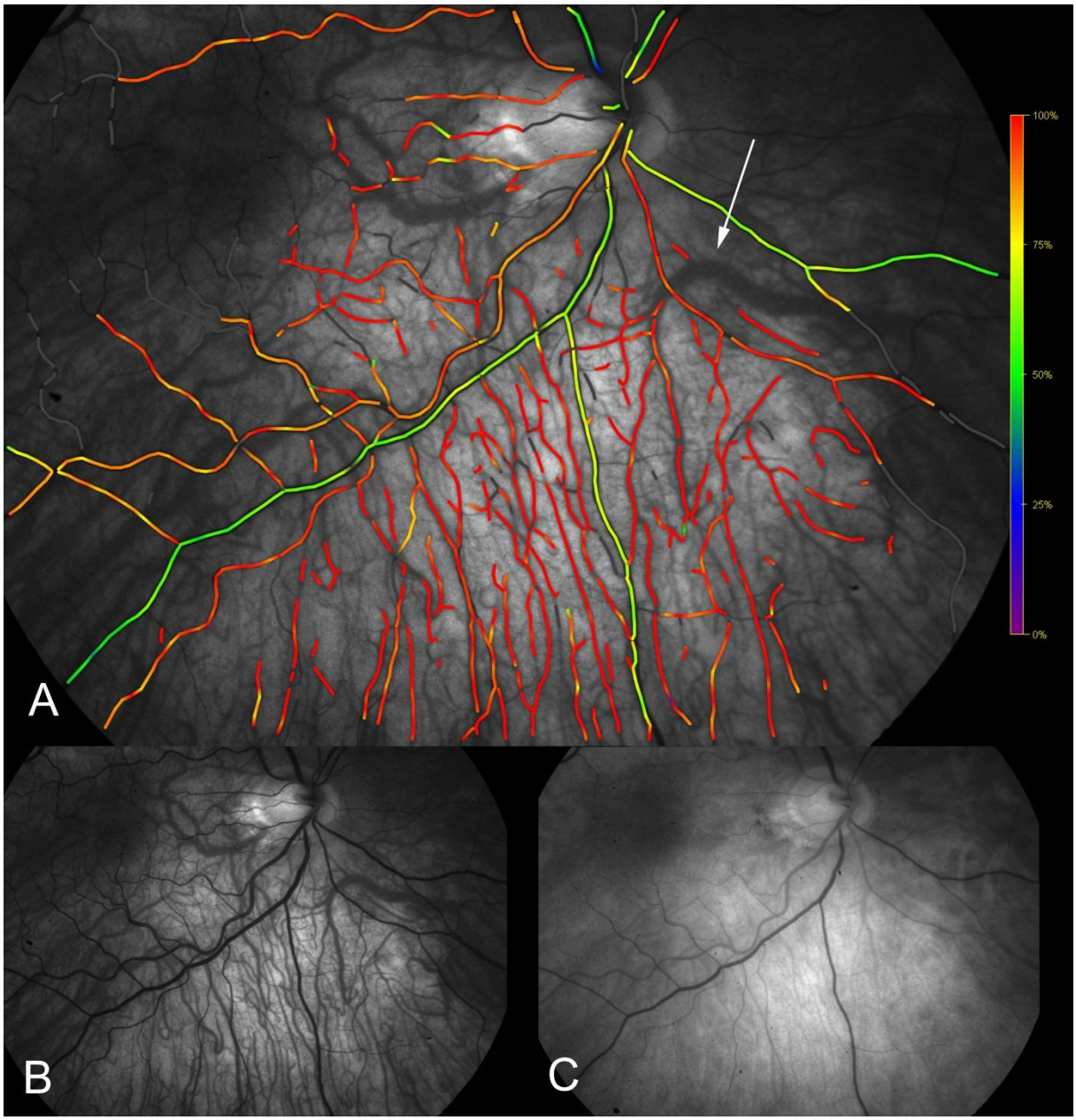

Figure 10. Choroidal oximetry image.

A. The vascular bed of the choroid is visible and measureable in lightly pigmented individuals with a spectrophotometric oximeter (Oxymap T1). Oxygen saturation in the vessels is colour coded. Red represents the highest oxygen saturation and the violet is the lowest (see scale on right side). Arrow is pointing at a visible choroidal vortex vein. **B.** Choroidal oximetry fundus image taken with an oxygen insensitive wavelength (570 nm). **C.** The same area of the fundus is captured simultaneously at an oxygen sensitive wavelength (600 nm). Figure reprinted from article: Kristjansdottir et al. (65) with permission from © Association for Research in Vision and Ophthalmology.

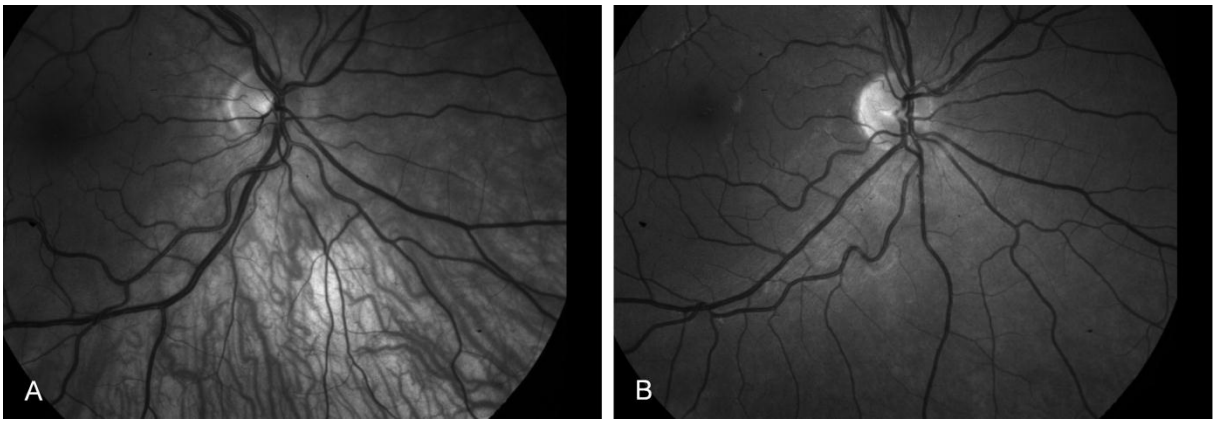

Figure 11. Lightly pigmented fundus vs. pigmented.

Comparison between **A.** lightly pigmented fundus with visible choroidal vasculature and **B.** pigmented fundus with poorly visible choroidal vasculature. The 16 subjects in the choroidal study were selected for the study from 120 participants in a normative study solely because the choroidal vasculature was visible and measureable with the retinal oximeter, Oxymap T1. Figure reprinted from article: Kristjansdottir et al. (65) with permission from © Association for Research in Vision and Ophthalmology.

All subjects went through the same standard study and imaging protocol. At first, all subjects answered a questionnaire on medication, medical history and smoking. Blood pressure and heart rate were measured (Omron M6 Comfort (HEM-7000-E), Omron Healthcare Europe, Hoofddorp, The Netherlands). Finger pulse oximetry (OhmedaBiox 3700, Ohmeda, Boulder, CO, USA), visual acuity (Snellen chart) and intra-ocular pressure (iCare TAO1 Tonometer, TiolatOy, Helsinki, Finland) were also measured. Pupils were dilated with 1% tropicamide (Mydriacyl; S.A. Alcon-Couvreur N.V., Puurs, Belgium), in some cases supplemented with 10% phenylephrine hydrochloride (AK-Dilate; Akorn Inc., Lake Forest, IL). All subjects were examined by a senior ophthalmology specialist and a 50 degree colour fundus image was taken of both eyes (Zeiss FF 450plus fundus camera, Carl Zeiss Meditec AG, Jena, Germany). After this, subjects underwent retinal oximetry measurement.

For retinal oximetry, five fundus images where acquired of both eyes of each subject using Oxymap T1. Images were acquired with four different angles of gaze: 1. macula centred, 2. optic disc centred, 3. optic disc down (superior fundus), 4. optic disc up (inferior fundus) and 5. optic disc centred (Fig. 12). All images were taken in a dark room; the only light source was the fundus camera and the computer screen. Fundus camera setting was standardised. The aiming light was at the lowest setting, which allowed a good view of the fundus and right alignment of the fundus camera. The fundus camera was set at a 50 degree field of view, small aperture and flash intensity 50 Ws. If needed, the small pupil setting was on (always registered if used). The right eye was imaged first, then the left. Five images were acquired of each eye in most cases, more if image quality was insufficient. Time between flashes for the same eye was approximately 30 seconds.

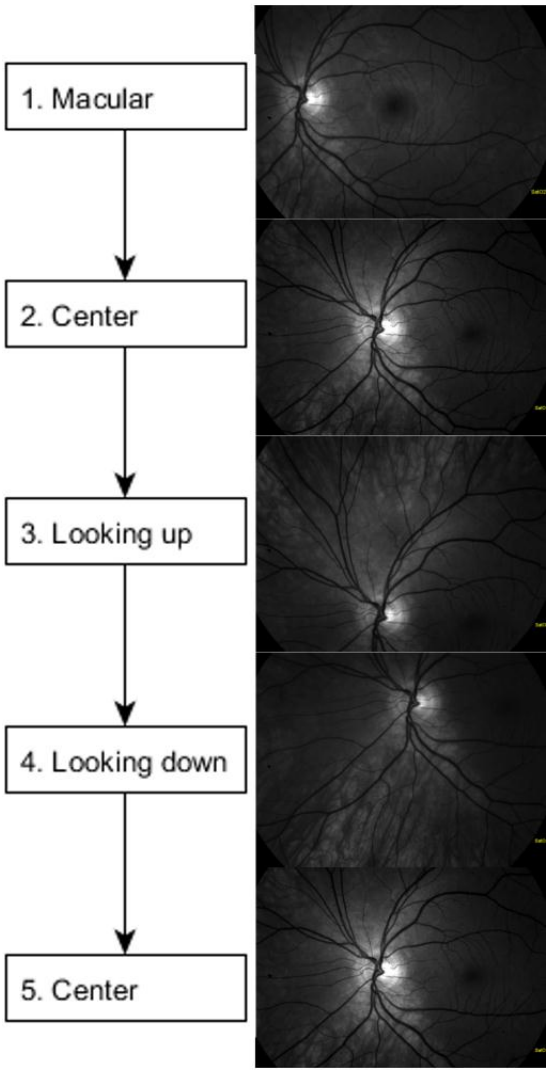

Figure 12. Imaging protocol.

Oximetry fundus images were acquired with four different angles of gaze: 1. macula centred, 2. optic disc centred, 3. optic disc down (superior fundus), 4. optic disc up (inferior fundus) and 5. optic disc centred (image 2 repeated).

3.1.4 Oximetry analysis

3.1.4.1 Normoxia

For the normoxia part of the study (n=16) image no. 4 in the imaging protocol was used for analysis (‘*4. Looking down*’, Fig. 12) because the choroidal vessels are best visible in the inferior fundus. The right eye was analysed in all cases except two because of poor image quality. ODR was measured for six segments of choroidal vessels (a mix of arterioles and venules), one segment of a choroidal vortex vein, one segment of a retinal arteriole and one segment of a retinal venule. The six choroidal vessel segments were averaged for each subject to get a good average of the area where the choroidal vessels were best visible and also so that the measurement would cover a similar area as the retinal arteriole and venule measured for each subject. All measured vessel segments (of all categories) were above 50 pixels in length and above diameter of 8 pixels (approximately 74 µm (66)).

Measurements were not continued beyond branching points. Otherwise, no upper limits were used for length of vessels or diameter. Because most visible choroidal vessels look alike and it cannot be determined whether they are arterioles or venules they are simply called choroidal vessels. The vortex veins in the choroid are recognized by their vortex pattern and wide diameter as indicated with an arrow in figure 10. Only 12 out of 16 subjects had visible and measurable vortex veins in the inferonasal quadrant of the fundus.

3.1.4.2 Repeatability

To test repeatability of the choroidal measurement, the left eye was analyzed using the same criteria as was used for the right eye. Three subjects were excluded, two because of poor image quality and one because the left eye did not have six visible and measureable choroidal segments.

3.1.4.3 Hyperoxia

Six of these 16 subjects (two males and four females, age of 42±19 years (mean±SD) also participated in a hyperoxia study where they inhaled pure oxygen (100% O_2) for 10 minutes (6 L/min, mask covering mouth and nose). Retinal oximetry images were obtained before and immediately after inhalation. Images were analysed the same way as before except for the choroidal vortex veins which were not visible in the hyperoxia part of the study because images were acquired with different angle of gaze, i.e. optic disc was centred (*´5. Center´*, Fig. 12).

3.1.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software Inc., LaJolla, CA, USA). A paired t-test was applied to compare ODR values for choroidal vessels and retinal arterioles and to compare normoxia and hyperoxia in all categories of measured vessels. Standard deviation between two repeated measurements of the same vessel was calculated using the square root of the within subjects mean square from ANOVA as recommended by Bland (67).

3.2 Scanning laser oximetry (paper II)

Optomap 200Tx is an ultra-wide field scanning laser ophthalmoscope (SLO) developed by Optos plc. (Dunfermline, Scotland, UK). The SLO uses two lasers, 532 nm (green) and 633 nm (red) to capture either 100 or 200 degree images of the retina in single capture.

Images from the SLO could, in principle, be used for dual wavelength retinal oximetry because 633 nm is non-isosbestic and 532 nm is close to being isosbestic (Fig. 3). By looking at figure 3, we see that the reference wavelength, 532 nm, is not completely isosbestic. The differences between light absorptivity of oxy- and deoxyhaemoglobin of 532 versus 633 nm is considerable and because of that, these wavelengths should work for oximetry. For 532 nm the ratio between light absorptivity of deoxyhaemoglobin (HB) and oxyhaemoglobin (HbO_2) is 0.7 while the ratio for 633 nm is 8.1, i.e. the Hb absorptivity at 633 nm is 8 times what it is for HbO_2 and the absorptivity of Hb at 532 nm is only 0.7

times that of HbO_2. These light absorptivity values of Hb and HbO_2 are according to results by Zijlstra et al.(18).

ODR was calculated according to equation 4 (p. 22) with the use of current wavelengths:

$$ODR = \frac{OD_{633}}{OD_{532}} \qquad eq.8$$

The two spectral images were processed by the Oxymap Analyzer software. The vessel detection algorithm of Oxymap Analyzer software (version 5206) was modified in order to process the larger images of the SLO and take into account the different zoom factor compared with the Oxymap T1 fundus-camera-based system.

Constants, a and b of equation 5 (p. 22), were found by matching the average arteriolar and venular ODRs from the healthy subjects with retinal oxygen saturation measurements performed with a calibrated device in a separate study by Schweitzer et al. (8). This resulted in following values: a=-2.4733 and b=1.4388.

Hereafter, the Optomap and Oxymap Analyzer combination will be referred to as the *SLO oximetry system*.

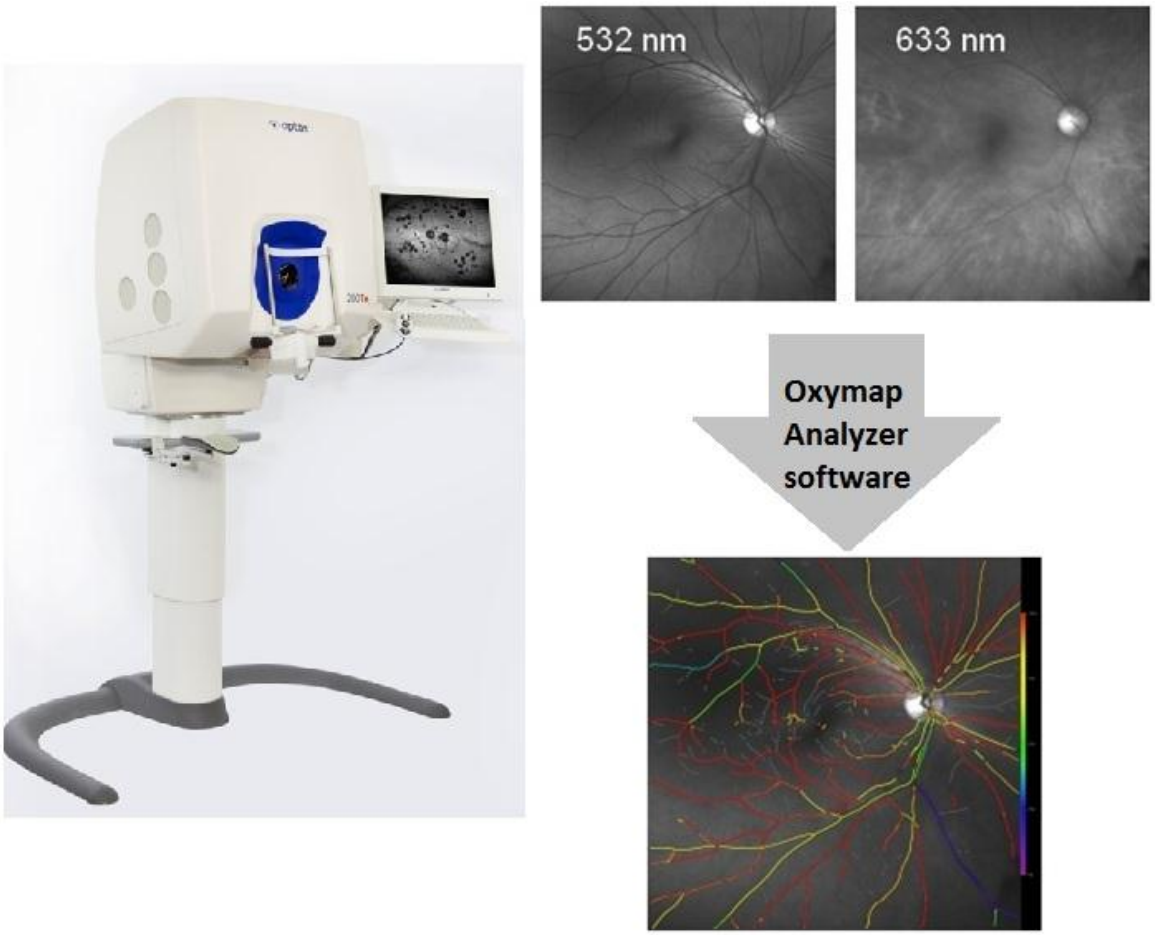

Figure 13. Scanning laser ophthalmoscope, Optomap 200Tx.

Optomap is an ultra-wide field scanning laser ophthalmoscope and acquires images of the fundus using two lasers (532 and 633 nm). With modified Oxymap Analyzer software, haemoglobin oxygen saturation of retinal vessels can be calculated.

3.2.1 Study protocol

3.2.1.1 Feasibility and repeatability

3.2.1.1.1 Normoxia

For initial adaption and testing of the SLO as a retinal oximeter, two 100 degree (ResMax setting) fundus images were acquired from eleven healthy volunteers (six men and five females); age 34±10 years (mean±SD). All subjects were recruited from the staff and faculty of the Ophthalmology department at Landspitali - University Hospital, Reykjavík, Iceland, and were normal healthy individuals with no history of ocular disease or trauma. Images were acquired of an un-dilated right eye of each subject. The SLO was set to store un-scaled 12 bit images and red and green sensors set at gain 2 (lightly pigmented iris). The first image from each subject was used for initial assessment of the device as a retinal oximeter, to test if there was a clear difference between arterioles and venules. Two images were acquired of the right eye of each subject to test the repeatability of measurements.

3.2.1.2 Sensitivity

Two different methods were used to test the sensitivity of the SLO for changes in haemoglobin oxygen saturation, pure oxygen inhalation, hyperoxia, and measurements of patients with confirmed retinal venous hypoxia due to either central retinal vein occlusion (CRVO) or hemivein occlusion.

3.2.1.2.1 Hyperoxia:

Two healthy subjects were measured before and after inhalation of pure oxygen (100% O_2) for 10 minutes (10 L/min), mask covering both mouth and nose. Images were acquired before oxygen inhalation started, when oxygen inhalation ended and every 5 seconds for the next 135 seconds during recovery and then finally, after 10 minutes of recovery. Before inhalation of pure oxygen started and during recovery phase, subjects breathed room air (21% O_2).

3.2.1.2.2 Hypoxia:

Three patients with CRVO and one patient with hemivein occlusion were also measured. All patients were recruited at the Department of Ophthalmology. Retinal venular hypoxia had previously been confirmed with Oxymap T1 oximeter (68). The CRVO affected eye was compared to the fellow eye. For the patient with hemivein occlusion the affected area (inferior fundus) was compared to the same area in the fellow eye.

3.2.2 Oximetry analysis

All oximetry images were analysed in a standardized manner. Normoxia and hyperoxia images of healthy individuals were all analysed in the same way; oxygen saturation was measured in the main superotemporal vessel pair, retinal arteriole and venule. For CRVO patients, all major retinal arterioles and venules above 6 pixels (approximately 60 µm) in diameter were measured. Retinal vessels with diameter smaller than that were excluded because saturation measurement of small retinal vessels can be unreliable.

3.2.3 Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.03. Paired t-test was applied to test the difference between arterioles and venules and difference between CRVO affected eye and healthy fellow eye. One-way ANOVA was used to test repeatability (67).

4 Results

4.1 Choroidal oximetry (paper I)

4.1.1 Normoxia

The mean optical density ratio (ODR) for 16 healthy individuals at a normal oxygen breathing condition (21% O_2) was 0.10±0.10 (mean±SD) for choroidal vessels, 0.13±0.12 for choroidal vortex veins, 0.22±0.04 for retinal arterioles and 0.50±0.09 for retinal venules (Fig. 14). According to a paired t-test the difference between choroidal vessels and retinal arterioles was statistically significant (p=0.0012, n=16) but difference between choroidal vessels and vortex veins was not statistically significant (p=0.175, n=12).

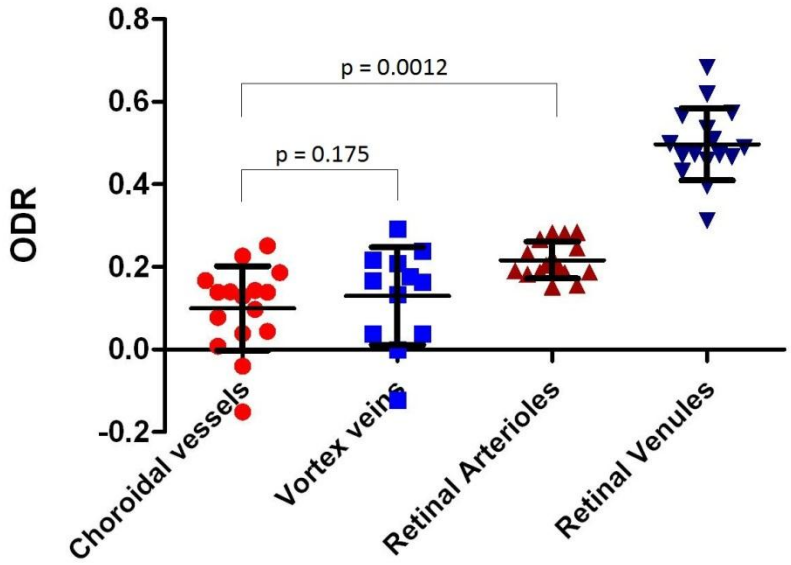

Figure 14. Optical density ratio (ODR) during normoxia.

Mean ODR, which is inversely related to haemoglobin oxygen saturation, is shown for choroidal vessels, vortex veins, retinal arterioles and retinal venules under normal oxygen breathing condition. Bars show one standard deviation.

4.1.2 Repeatability

The difference in ODR between the right and the left eye (choroidal vessels) was not significant (p=0.14, paired t-test). Standard deviation between choroidal ODR measurements of the right and left eye in the same individual was 0.07 ODR units (Fig. 15).

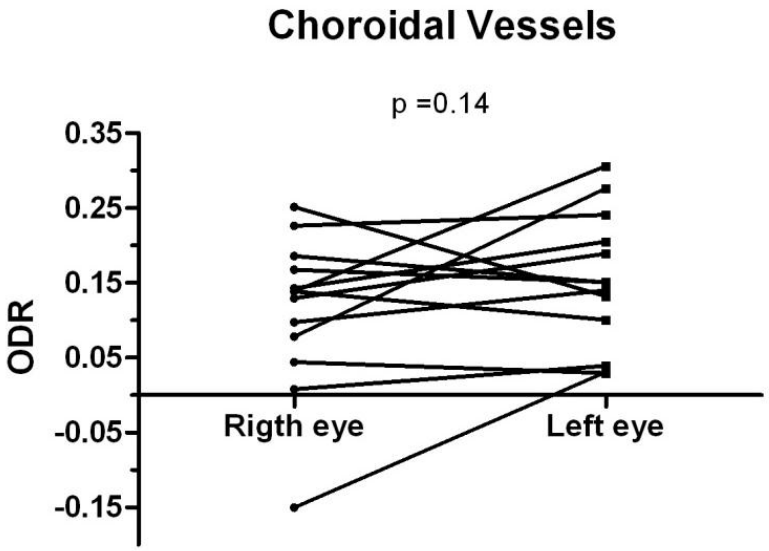

Figure 15. Repeatability of choroidal vessel measurement. Right vs. left eye.

For repeatability testing the left eye was measured using the same criteria as was used for the right eye. According to a paired t-test the difference was not significant (p=0.14). Standard deviation between measurements of the right and left eye in the same individual was 0.07 ODR units. Figure reprinted from article: Kristjansdottir et al. (65) with permission from © Association for Research in Vision and Ophthalmology.

4.1.3 Hyperoxia

Inhalation of pure oxygen (100% O_2) (n=6) lowered ODR levels in choroidal vessels, retinal arterioles and retinal venules. The decrease in ODR between normoxia and hyperoxia was statistically significant for all vessel types (Fig. 16). The decrease was 0.035±0.028 for choroidal vessels (p=0.028, paired t-test), 0.022±0.017 for retinal arterioles (p=0.022, paired t-test) and 0.246±0.067 for retinal venules (p=0.0003, paired t-test). In addition, the difference between choroidal vessels and retinal arterioles remained statistically significant during hyperoxia (p=0.021, paired t-test).

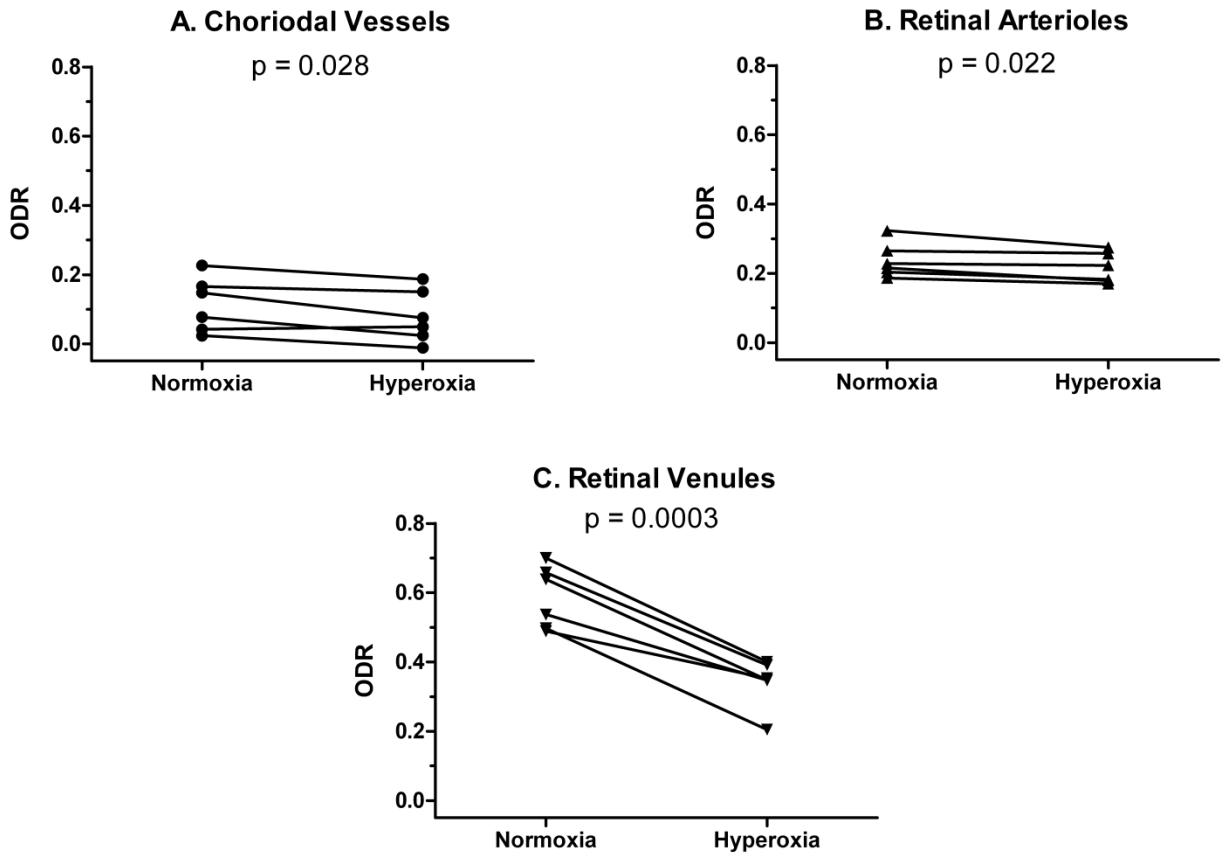

Figure 16. Optical density ratio (ODR) during normoxia and hyperoxia.

ODR is inversely related to haemoglobin oxygen saturation. **A.** choroidal vessels, **B.** retinal arterioles and **C.** retinal venules under normal oxygen breathing condition (normoxia) and with subjects breathing pure oxygen (hyperoxia). Figure reprinted from article: Kristjansdottir et al. (65) with permission from © Association for Research in Vision and Ophthalmology.

4.2 Scanning laser oximetry (paper II)

4.2.1 Feasibility and repeatability

4.2.1.1 Normoxia

Oxygen saturation was 92%±13% (n=11, mean±SD) for arterioles and 57%±12% for venules (mean saturation values are a direct result of calibration). The difference between arterioles and venules was statistically significant according to a paired t-test (p=0.0001). Standard deviation for repeated measurements of the same vessel was 3.5% for arterioles and 4.4% for venules. Figure 17 highlights the difference between arteriolar and venular saturation in each individual. Figure 18 shows the repeatability between measurements of two images from each individual.

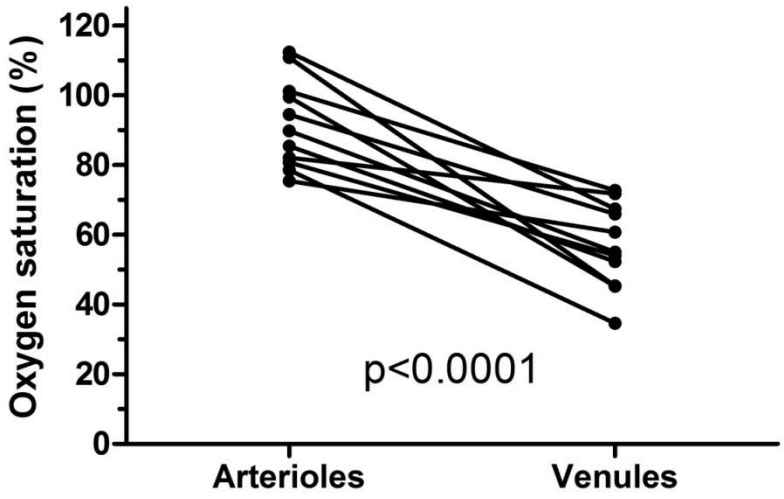

Figure 17. Oxygen saturation of retinal arterioles and venules.

The graph shows oxygen saturation of the main superotemporal vessel pair (arteriole and venule) for 11 healthy subjects. Lines connect vessels in the same eye.

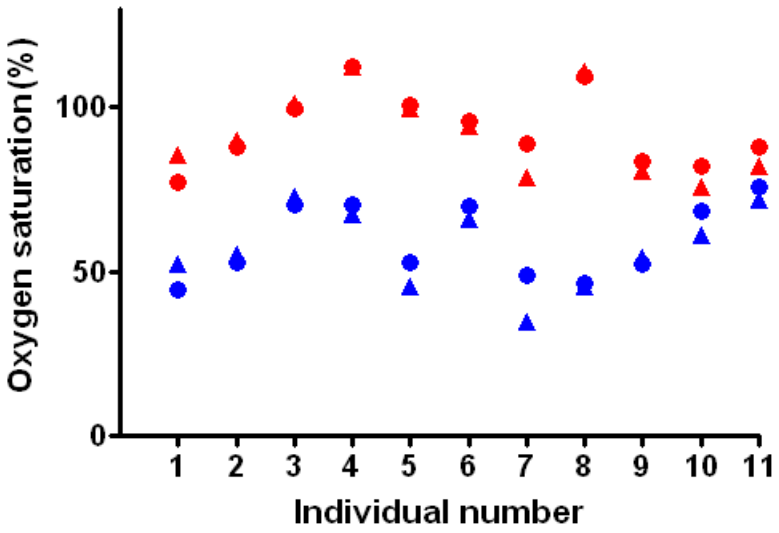

Figure 18. Repeatability of the SLO oximetry system.

The graph shows oxygen saturation of the main superotemporal vessel pair (arteriole and venule) from 11 healthy subjects. Two images were analysed for each subject. Triangle denotes first image for each individual and the circle denotes second image. (Red for arterioles and blue for venules). Standard deviation for repeated measurements of the same vessel was 3.5% for arterioles and 4.4% for venules.

4.2.2 Sensitivity

4.2.2.1 Hyperoxia

Figures 19 and 20 show results of pure oxygen breathing experiment. This is a sensitivity test of the SLO oximetry system for haemoglobin oxygen saturation measurements. Figure 19 shows oxygen saturation over time during recovery from pure oxygen inhalation. Figure 20 shows an example of how the pseudocolour map of retinal oxygen saturation changes with breathing of pure oxygen.

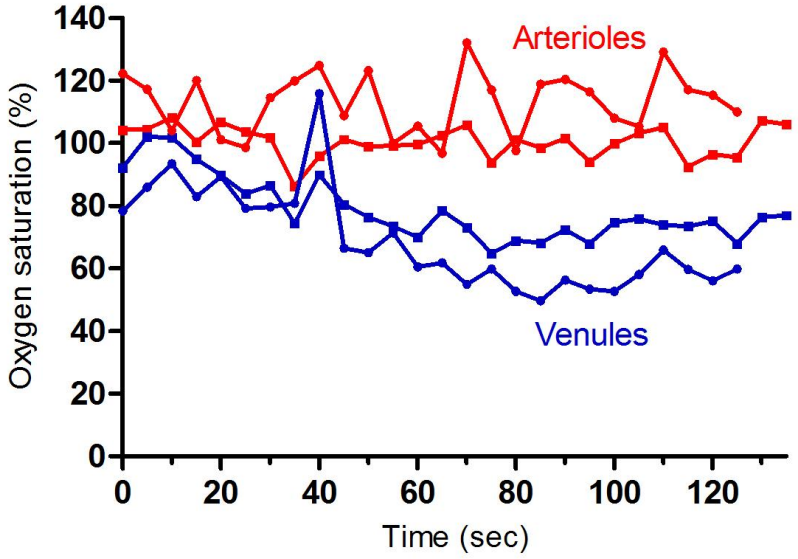

Figure 19. Oxygen saturation over time. Recovery from pure oxygen inhalation.

Haemoglobin oxygen saturation over time for retinal arterioles (red) and venules (blue) for two healthy subjects (Subject 1 denoted with *circles*, subject 2 denoted with *squares*). Subjects inhaled pure oxygen for 10 minutes (10 L/min) and fundus images were acquired with Optomap 200Tx after the inhalation ended (time=0) and every 5 seconds for 135 seconds during recovery (breathing of room air, 21% O_2). Oxygen saturation was analysed using Oxymap Analyzer software.

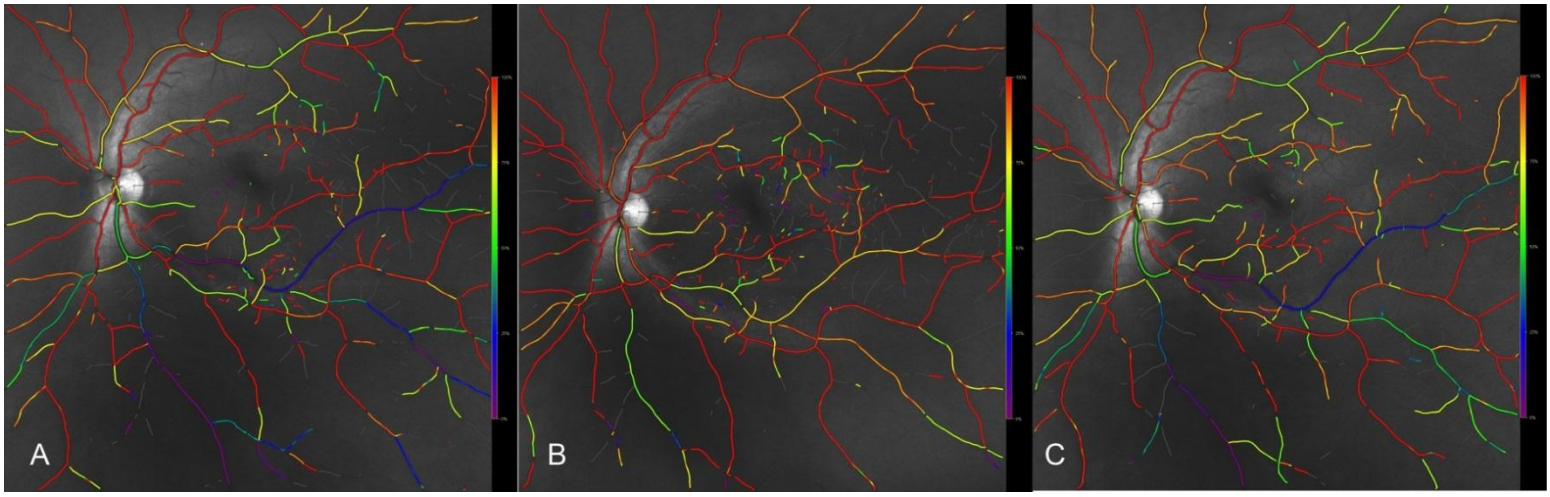

Figure 20. Inhalation of pure oxygen. Pseudocolour map changes with inspired oxygen.

Oximetry images from one healthy subject before and after inhalation of pure oxygen. **A.** Before inhalation started. **B.** After inhalation of pure oxygen for 10 minutes. **C.** After 10 minutes of recovery (inhalation of room air).

4.2.2.2 Hypoxia

Table 1 and figures 6 and 7 show results of measurements of patients with CRVO or hemivein occlusion.

Table 1. Oxygen saturation of CRVO/hemivein patients.

Oxygen saturation (mean±SD) of four patients with either central retinal vein occlusion (CRVO) (pat.1-3) or hemivein occlusion (pat. 4). Affected eye is compared to healthy fellow eye. Difference between retinal arterioles in affected eyes and fellow eyes was not statistically significant (n=4, p=0.6405, paired t-test), difference between retinal venules in affected eyes and fellow eyes was statistically significant (n=4, p=0.0009, paired t-test)

Oxygen saturation (%) in retinal vessels (mean ± SD)				
	Affected eye		Fellow eye	
Pat.no.	Arterioles	Venules	Arterioles	Venules
1	109 ± 21	25 ± 42	110 ± 13	56 ± 5
2	108 ± 9	26 ± 26	101 ± 14	60 ± 22
3	115 ± 11	19 ± 13	116 ± 8	59 ± 24
4	95 ± 10	20 ± 28	109 ± 15	63 ± 7
Mean	107 ± 9	23 ± 3	109 ± 6	59 ± 3

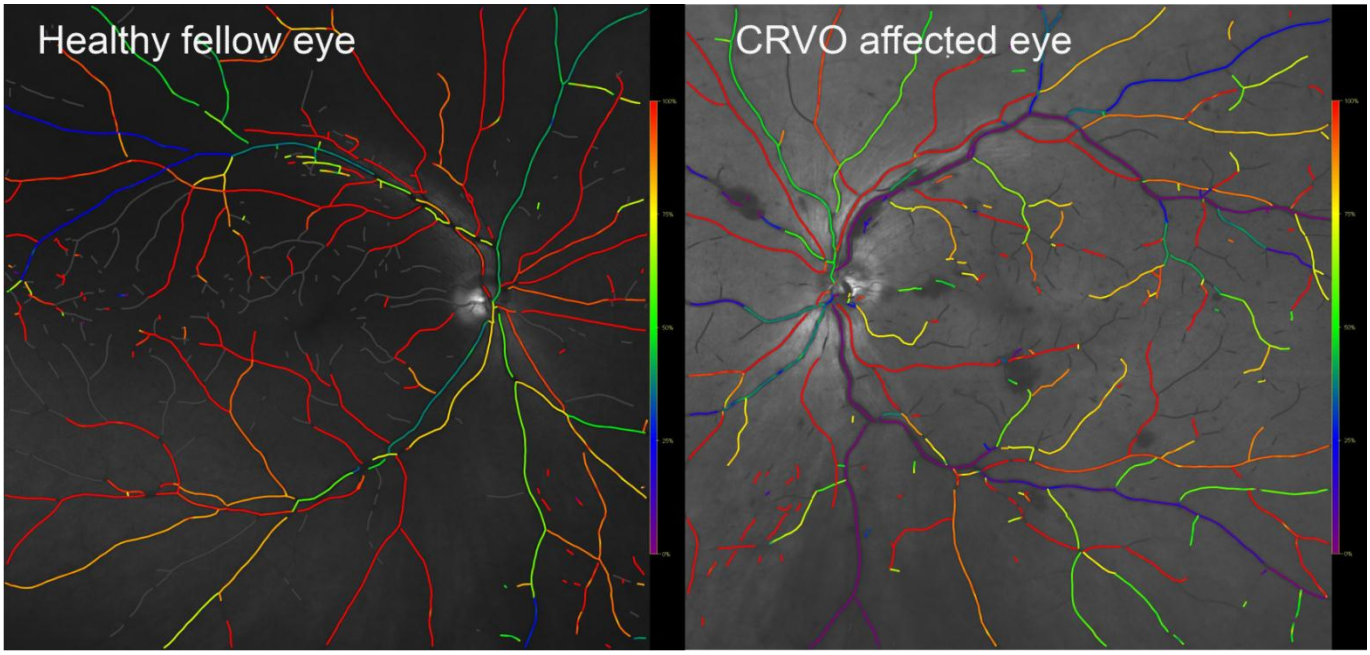

Figure 21. Oximetry images from a patient with central retinal vein occlusion (CRVO).

A clear difference is seen in venular oxygen saturation between CRVO affected eye and the healthy fellow eye (see scale on right side, red colour indicates 100% and violet indicates 0% oxygen saturation).

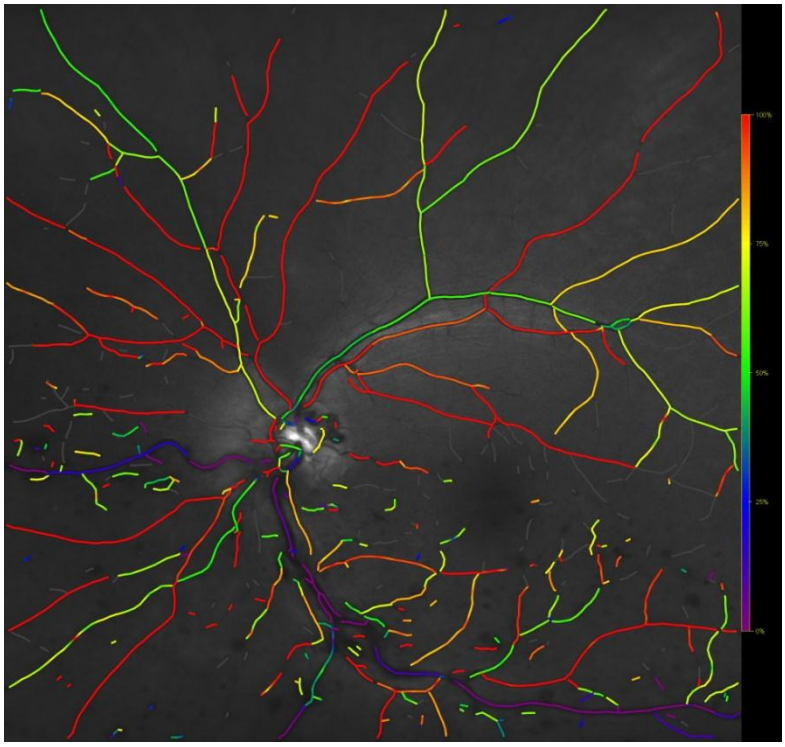

Figure 22. Oximetry images from a patient with inferior hemivein occlusion.

Inferior retinal venules are occluded and a clear difference is seen in oxygen saturation between superior and inferior venules, see scale on right side, red colour indicates 100% and violet indicates 0% oxygen saturation.

5 Discussion

5.1 Choroidal oximetry (paper I)

Optical density ratio (ODR), which correlates inversely with haemoglobin oxygen saturation, can be measured in the choroidal vasculature in lightly pigmented individuals with a spectrophotometric oximeter. Results show that the oximeter is sensitive to changes in choroidal and retinal vessel oxygen saturation due to pure oxygen (100% O_2) versus atmospheric air (21% O_2) inhalation.

Even though calculation of oxygen saturation from choroidal ODR has not been attempted, a low ODR is consistent with high oxygen saturation and high saturation in the choroid is in agreement with earlier studies on oxygenation in animals (55, 69). Shahidi et al. (55) used semi-invasive phosphorescence imaging system to measure oxygen tension (pO_2) in rat eyes and measured higher pO_2 in the choroid than retinal arterioles under normal and increased oxygen breathing condition, this agrees with our findings in the human eye. For comparison, the relationship between pO_2 and haemoglobin oxygen saturation can be determined using the oxygen-haemoglobin dissociation curve (18).

While measuring the choroidal vessel ODR is new in this field, measurements on the retinal arterioles and venules is not new and our measurements on the retinal vessels are also in good agreement with previous studies made using an automated image analysis technique based on dual-wavelength oximetry similar to our technique (24, 70).

According to published results by Alm and Bill, the arteriovenous difference in oxygen content in the cat choroid is only 3% (7). This agrees with our experience, from our choroidal measurements, that choroidal arterioles and venules are difficult to distinguish with spectrophotometric oximetry and that choroidal vortex veins only have slightly higher ODR. Even though difference is not statistically significant, there is an indication of slightly lower oxygen saturation (higher ODR) in the vortex veins compared to other choroidal vessels (Fig. 14).

The repeatability of retinal oxygen measurements with the oximeter has been determined previously (47). The standard deviation of repeated measurements was 1.0% for arterioles and 1.4% for venules. Repeatability of the choroidal measurement was not tested in the same way in this study but can nonetheless be estimated. We calculated the standard deviation between choroidal ODR measurements of left and right eye in the same individual. This is displayed in ODR values in the results and if ODR values are transformed into saturation (with standard Oxymap T1 retinal calibration), the standard deviation between measurements of the left and right eye in the same individual is 7%. Although the study was not designed to estimate repeatability, these values indicate that the variability is greater for the choroidal measurements than it is for retinal measurements.

By using the standard Oxymap T1 retinal calibration for the choroidal vessel ODR, the calculated haemoglobin oxygen saturation is 107±12% (mean±SD) for the choroidal vessels, 106±13% for the vortex veins, 94±5% for retinal arterioles and 59±9% for retinal venules (see ODR in figure 14). This calibration is apparently not appropriate for the vessels within the choroid where oxygen saturation exceeds 100%. For the retinal vessels, mean oxygen saturation is about 2% higher, for both arterioles and venules for the lightly pigmented subgroup (choroidal vessels visible) compared to the group of

120 healthy individuals, where mean arteriolar saturation was 92%±4% and venular saturation was 57%±6% (46). Subjects for this study were selected because of the fact that the choroidal vasculature was visible and measureable due to light pigmentation and the higher measured retinal oxygen saturation may be because of this difference in pigmentation. The measurement of retinal oxygen saturation is dependent on fundus pigmentation to some degree. This has been previously confirmed with similar retinal oximeters as used for current study (24, 27).

The optical properties of the intervascular tissue in the choroid may play a role in the choroidal vessel measurements. The ODR was lower in the choroidal vessels than in retinal arterioles, potentially indicating higher oxygen saturation in the choroid. However, it is also observed that the optical density (OD) of choroidal vessels is reduced at both 570 and 600 nm, which can affect the ODR and oxygen saturation conversion. This is compatible with scattering of light within the choroid reducing the contrast of choroidal vessels as follows. The OD of retinal vessels is measured against a bright background dominated by scattering from interstitial tissue. Choroidal blood vessels are embedded within this interstitial tissue and components of this tissue lying between blood vessels and the retinal pigment epithelium backscatter incident illumination, which reduces contrast of choroidal vessels; that is, it reduces OD. The magnitude of the scattering from interstitial tissue is approximately equal at both 570 and 600 nm, but causes a proportionately greater reduction in the OD for the lower OD measurements at 600 nm. In consequence, there is a reduction in the ODR for choroidal vessels. If the reduction in ODR due to scattering is neglected, this would imply higher oxygenation levels and suggests non-realistic oxygen saturation in excess of 100% in choroidal vessels. It is probable that the different illumination of the vessel by the surrounding choroidal tissue also has an effect, though this is expected to be less significant.

Inhalation of pure oxygen (hyperoxia) lowered the ODR levels for all measured vessel types, both choroidal and retinal, which corresponds to an increase in oxygen saturation. By using the standard Oxymap T1 retinal calibration, the increase, in haemoglobin percentage points, was found to be 4% for choroidal vessels, 2% for retinal arterioles and 26% for the retinal venules. The reason for this large increase in retinal venular oxygen saturation is that with hyperoxia a larger amount of oxygen diffuses from choroid to the retina due to increased oxygen gradient. This reduces extraction of oxygen from the retinal vasculature and therefore increases the saturation in the retinal venules (71). The retinal saturation measurement is in good agreement with previous studies using the Oxymap T1 (25, 44) and other dual-wavelength retinal oximeters (27, 70) during hyperoxia but measurements have not been done on choroidal vessels until now. The significant increase in all measured vessel types due to pure oxygen inhalation demonstrates that Oxymap T1 oximeter is sensitive to changes in oxygen saturation for both the choroidal and retinal vessels (Fig. 16, vortex veins were not visible on images taken for the hyperoxia part of the study).

Different optical properties of the retinal and choroidal vessel measurements result from the fact that retinal and choroidal vessels lie in different tissues at different depth. The result is that the standard calibration, which has been used for retinal vessels to transform ODR into oxygen saturation, is not appropriate for choroidal vessels. However, the lowering of ODR in the choroidal vessels with

hyperoxia demonstrates that the oximeter is sensitive to changes in oxygen saturation in choroidal vessels as well as in retinal vessels.

We measured only individuals with the most visible choroidal vessels. These were only 16 individuals from a group of 120. Thereof, only six were available for hyperoxia. The small sample sizes may make the parametric statistical tests used vulnerable to deviations of the population from normal distribution. We therefore re-calculated all p-values using the Wilcoxon signed rank test. This did not change the conclusions of the study, although the difference between normoxia and hyperoxia in choroidal vessels became borderline statistically significant ($p=0.063$) and the same is true for the comparison of retinal arterioles and choroidal vessels during hyperoxia ($p=0.059$).

It is furthermore observed that for some of the imaged retinas the OD of choroidal vessels imaged at 600 nm is negative; that is, vessels appear brighter than the surrounding choroidal tissue and this leads to the negative ODRs shown in figure 14. Study of these images suggests that this brightness is associated with diffuse structure in the scattering interstitial tissue that correlates with the vessel structure and that there is insufficient contrast to detect an OD due to the vessel. This may be because the vessels are located more deeply within the choroidal tissue than those vessels for which positive ODs can be measured. In these cases the ODR for these vessels is effectively zero.

The physical optics leading to the observed ODR of choroidal vessels is inherently different from that underpinning oximetry of retinal vessels. The determination of choroidal vessel oxygenation will require some modification and refinement to the physical optics model established for retinal vessel oximetry and this is the subject of on-going investigation. It is clear, however, from these results that it is nevertheless possible to detect changes in choroidal vessel oxygenation associated with changes in inspired oxygen.

5.2 Scanning laser oximetry (paper II)

The combination of Optomap 200Tx SLO imaging and Oxymap Analyzer software was successful in the development of an SLO based retinal oximetry system. Results indicate that haemoglobin oxygen saturation can be measured using this combination. Measurements are repeatable and SLO oximetry system is sensitive to changes in oxygen saturation due to pure oxygen inhalation or retinal venular hypoxia.

The initial test of whether the SLO could work as an oximeter was successful. There was a measurable difference between arterioles and venules (Fig. 17).

The repeatability of the measurement is good as indicated by the standard deviation for repeated measures (Fig. 18), 3.5% for arterioles and 4.4% for venules. This is acceptable considering the early stage in the development. The more developed fundus-camera-based system, Oxymap T1, has standard deviation for repeated measures of 1.0% for arterioles and 1.4% for venules (47). There are no published results on repeatability of other SLO oximeters.

The SLO oximetry system is sensitive to different oxygen saturation in retinal vessels. This can first of all be seen from the clear difference measured between arterioles and venules (Fig. 17). Second of

all, the system is sensitive for changes in oxygen saturation during pure oxygen breathing and changes in oxygen saturation over time (Figs. 19 and 20). From figure 19 we can see that the oxygen saturation for retinal arterioles stays relatively stable over time while the saturation for retinal venules decreases, for both subjects (A and B). These results are in good agreement to previously published results using the Oxymap T1 system (Fig. 23) (44). Even though, we can see by comparing the graphs that the variability in measurements is considerably greater for the SLO oximetry system compared to the Oxymap T1. We see more fluctuations in the SLO oxygen saturation measurements.

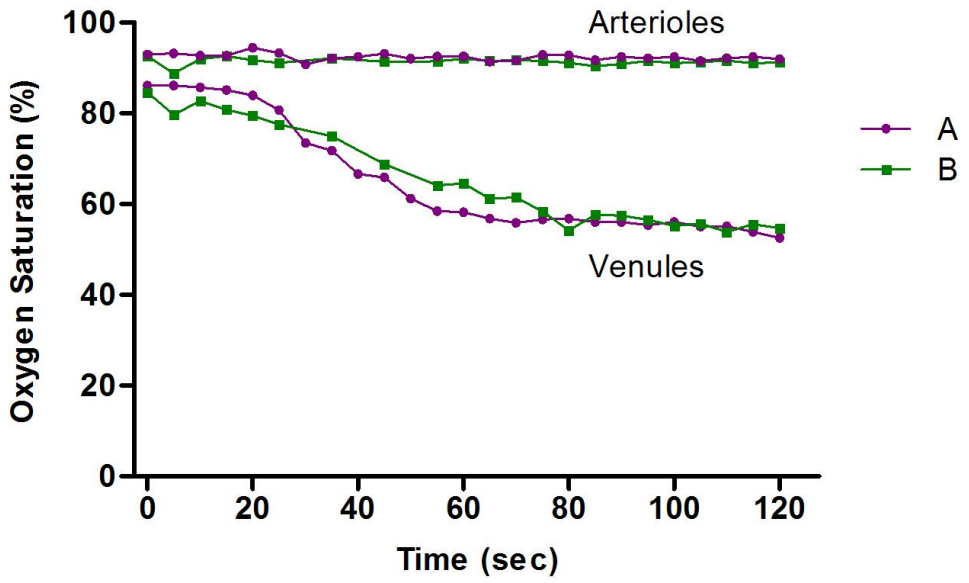

Figure 23. Oxygen saturation over time for retinal arterioles and venules for two healthy subjects (Oxymap T1).

Subjects (A and B) inhaled pure oxygen for 10 minutes (6 L/min) and fundus images were acquired with the Oxymap T1 oximeter after the inhalation ended (time=0) and every 5 seconds for 120 seconds during recovery (breathing of room air). Oxygen saturation was analysed using Oxymap Analyzer software. Graph from different study done by Olafsdottir et al. (44).

The sensitivity of the system can also be seen in retinal vein occlusion cases. The SLO oximetry system clearly detects the difference between oxygen saturation in CRVO/hemivein affected eyes and healthy fellow eyes (Table 1, Figs. 21 and 22), the difference is statistically significant for retinal venules. The venular hypoxia had already been confirmed in all of these subjects with the use of the more developed Oxymap T1 oximeter (68). The SLO oximetry images do also provide picturesque wide field images of the variable hypoxia in CRVO/hemivein occlusion, which could be clinically useful.

The main difficulty of the SLO oximetry system is the considerable variability in oximetry measurements. There is both intra-eye (within eyes) and inter-eye (between eyes) variability. Intra-eye variability is between vessels of the same eye and an example of this can be seen in the healthy eye

in figure 20, where several vessel segments show abnormally low saturation (see enlarged colour scale on Fig. 9). Normal retinal oximetry for a healthy individual is orange to red for arterioles (90-100% $SatO_2$) and blue to green for venules (50-60% $SatO_2$) as has been confirmed with Oxymap T1 (Fig. 9) (46). The variability between eyes or individuals is also considerable as evidenced by the standard deviations for the group of 11 healthy individuals, 13% for arterioles and 12% venules (Fig. 17). This variability is greater than seen with Oxymap T1 system today (46, 47) but still similar to the variability seen in early days of developing the Oxymap T1 system (25). Technical optimisation in the development process proved successful in reducing the variability of measurements with the Oxymap T1 system and similar improvements can be expected with SLO oximetry with future studies. To improve the SLO oximetry system, technical changes to the Optomap must be made such as trying different laser wavelengths for imaging. Those kinds of tests require hardware changes to the Optomap and should be the work of future studies.

One of the advantages of the SLO oximetry system is the wide field of view of the retina imaged in single capture. Optomap can acquire both 100 and 200 degree images of the retina. In the current study, 100 degree images were used for oximetry and oxygen saturation was mapped of all the retinal vessels of the image. SLO oximetry images could also be produced with the 200 degree wide field images but the image quality of the peripheral fundus images from Optomap is still quite poor and that makes oximetry at this wide angle more difficult. Also, the Oxymap software would need further modification for these extra wide field images. With further development of the wide field imaging of the Optomap, oximetry analysis of the peripheral fundus might get reliable. Peripheral oximetry mapping can be useful in various eye diseases e.g. diabetic retinopathy. Other published studies using SLO for retinal oximetry did not manage to image the retina at this large angle as our SLO oximetry system, only a small area of the retina was imaged, either one vessel or one vessel pair. Results published by Smith et al. (63), Drewes et al. (34) and Denninghoff et al. (64) show retinal vessel oximetry measurements across a slit of small area of the retina and haemoglobin oxygen saturation calculated from a one-dimensional intensity profiles generated from scans. Ashman et al. (33) also used similar method, oxygen saturation only measured from a single slit over one arteriole and one venule. Li et al. (32) used adaptive optics to measure small vessels (< 50 µm), also in a very small area at a time.

Other advantages of the SLO are that no dilation of the pupil is needed and imaging through cataracts and other opacities is easier than with regular fundus photography. It is important to be able to image without being dependent on dilation in cases where patients' pupils do not dilate enough e.g. in some elderly people and people with diabetes. Elderly people often have cataracts and/or some other optical opacity and they are most often the group of interest for retinal oximetry, due to the age-dependent prevalence of many common and serious eye diseases.

6 Conclusion

6.1 Choroidal oximetry (paper I)

Choroidal vessel optical density ratio (ODR) can be measured in people with little pigment in the fundus. Oxymap T1 oximeter clearly detects a signal from the choroidal vessels and the difference between normoxia and hyperoxia is statistically significant. ODR is inversely related to haemoglobin oxygen saturation and using retinal vessel calibration for choroidal vessels gives non-realistic saturation values exceeding 100%. The retinal calibration might not be appropriate for choroidal vessels and needs to be adapted to the choroidal measurement. When calibrating for the choroidal vessels the different scattering properties of the choroid need to be taken into account and the solution might be a scattering correction factor. This is a work of future studies on choroidal oximetry.

Oxygenation of the choroidal vasculature is important in many major eye diseases and the interaction between the retinal and choroidal vasculatures is far from being fully known, at least in humans. For now, choroidal oximetry is only feasible in lightly pigmented individuals. Despite that, choroidal oximetry measurements in healthy and lightly pigmented individuals could give valuable information on the physiological behaviour of the choroid. It might be evaluated from lightly pigmented groups, such as the group studied in current study e.g. measure the effect of light and dark on choroidal oxygenation, effect of hypoxia or effect of systemic changes, such as increasing the blood pressure. This might further the understanding of the interaction between retinal and choroidal vasculatures, which might be helpful in understanding pathophysiology of some of the major eye diseases.

6.2 Scanning laser oximetry (paper II)

Combining scanning laser ophthalmoscope (SLO) imaging and retinal oximetry software was successful in developing a system for SLO oximetry, which is both sensitive and gives repeatable results. The intra- and inter-eye variability is still large and further hardware development is needed to decrease the variability. Optimisation may include trying different lasers for imaging and along with any hardware changes the software needs to be modified.

Given the advantages that SLO imaging has over conventional fundus camera optics in retinal oximetry, such as no dilation needed, larger field of view, fast and easy fundus photography, further development of SLO oximetry may provide the optimal approach to retinal oximetry.

SLO oximetry is promising, future steps in development should include technical changes to better reduce the great variability of the haemoglobin oxygen saturation measurement. Measuring the choroidal vessels is also of interest with the use of SLO imaging. The SLO used here, uses longer wavelengths which can reach the choroidal vessels more easily than the fundus camera. The aim of future studies should be that the SLO oximetry system will be useful for both the retina and the choroid.

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Appendix

A. Published article:

Choroidal oximetry with a noninvasive spectrophotometric oximeter.

Kristjansdottir JV, Hardarson SH, Harvey AR, Olafsdottir OB, Eliasdottir TS, Stefánsson E. Invest Ophthalmol Vis Sci. 2013 May 7;54(5):3234-9.

B. Article submitted, under review:

Retinal oximetry with a scanning laser ophthalmoscope

Kristjansdottir JV, Hardarson SH, Halldorsson GH, Karlsson RA, Eliasdottir TS, Stefánsson E.

Other published articles (not included in thesis):

Retinal oximetry images must be standardized. A methodological analysis. Palsson O, Geirsdottir A, Hardarson SH, Olafsdottir OB, **Kristjansdottir JV**, Stefánsson E. Invest Ophthalmol Vis Sci. 2012 Apr 2;53(4):1729-33.

Retinal vessel oxygen saturation in healthy individuals.

Geirsdottir A, Palsson O,Hardarson SH, Olafsdottir OB, **Kristjansdottir JV**, Stefánsson E. Invest Ophthalmol Vis Sci. 2012 Aug 13;53(9):5433-42.

Appendix A

Choroidal Oximetry With a Noninvasive Spectrophotometric Oximeter

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Purpose. The purpose of the study was to establish a new technology to measure hemoglobin oxygen saturation in human choroidal vasculature with a noninvasive spectrophotometric oximeter.

Methods. The fundus camera–based oximeter captures dual-wavelength oximetry images of the fundus and calculates optical density ratio (ODR), which is inversely related to hemoglobin oxygen saturation. Sixteen healthy and lightly pigmented individuals were imaged during normoxia and six during both normoxia and pure oxygen breathing (hyperoxia). ODR was measured for choroidal vessels, vortex veins, and retinal arterioles and venules.

Results. ODR was 0.10 ± 0.10 (mean ± SD) for choroidal vessels, 0.13 ± 0.12 for vortex veins, 0.22 ± 0.04 for retinal arterioles, and 0.50 ± 0.09 for retinal venules. Inhalation of pure oxygen lowered ODR levels in all vessel types; the decrease was 0.035 ± 0.028 in choroidal vessels ($P = 0.029$, paired t-test), 0.022 ± 0.017 in the retinal arterioles ($P = 0.022$, paired t-test), and 0.246 ± 0.067 in retinal venules ($P = 0.0003$, paired t-test).

Conclusions. The ODR can be measured noninvasively in the choroidal vessels of lightly pigmented individuals and is significantly lower in choroidal vessels than in retinal arterioles. This may suggest higher oxygen saturation but is also compatible with the reduced contrast of choroidal vessels at both wavelengths that is expected from scattering of light within the choroid. The decrease of ODR during hyperoxia was significant for all vessel types, which confirms that the oximeter is sensitive to changes in oxygen saturation in both choroidal and retinal vessels.

Keywords: oxygen, choroid, retina, optical density, hyperoxia

The human retina is dependent on two separate vascular systems, the retinal vessels and the choroid. Inner layers of the retina are served by the retinal vessels with oxygen and nutrition, while outer layers of the retina, including the highly metabolically active photoreceptors, are served mainly from the choroid. The choroid has extraordinarily high oxygen tension,[1] and the arteriovenous difference in oxygen content in the choroid is very low. Experiments on cats have demonstrated an arteriovenous difference in oxygen content of only 3%,[2] while it is around 35% in the retinal circulation.[3] Present knowledge of oxygenation in the choroid comes almost exclusively from invasive animal studies because lack of a safe and reliable noninvasive technology for oxygen studies in the choroid has prevented studies on humans until recently.

In 1961 Broadfoot et al.[4] reported a modified ophthalmoscope capable of noninvasively detecting changes in choroidal oxygen saturation in humans. This involved nonimaging measurement of the light reflected from the ocular fundus for illumination by light in four broad spectral bands during deep normal breathing and apnea. In 1975, Laing et al.[5] reported a nonimaging spectrophotometric choroidal oximeter that, following calibration, was able to continuously quantify choroidal oxygen saturation. It was composed of a fundus-monitoring unit, dual-wavelength (650 and 805 nm) light source and electrical system for synchronous processing of signals and calculation of the oxygen saturation. These choroidal oximetry studies establish the possibility of detecting differences in choroidal oxygenation in humans using noninvasive spectrophotometric methods. The technique used and described in this paper for spectrophotometric measurements of choroidal oxygen saturation is based on the same basic principles as Hickam et al.[6] and Beach et al.[7] used for calculating retinal oxygen saturation and was reported by Hardarson et al.[8] in 2006 and now recently by Geirsdottir et al.[9] Whereas previous measurements have determined oximetry averaged over an extended area of the choroid, we explore here the possibility of oximetric imaging of choroidal vessels.

Choroidal oxygen saturation measurements are important from at least two points of view. First, they may allow study of pathophysiology of diseases in which choroidal ischemia and abnormalities in choroidal blood flow may play a role, such as age-related macular degeneration,[10–12] diabetic retinopathy,[13,14] and central serous choroioretinopathy.[15] Second, they may allow direct measurements of oxygen saturation in central vasculature. This is important in cardiovascular shock and severe injury, in which peripheral oxygenation as measured, for

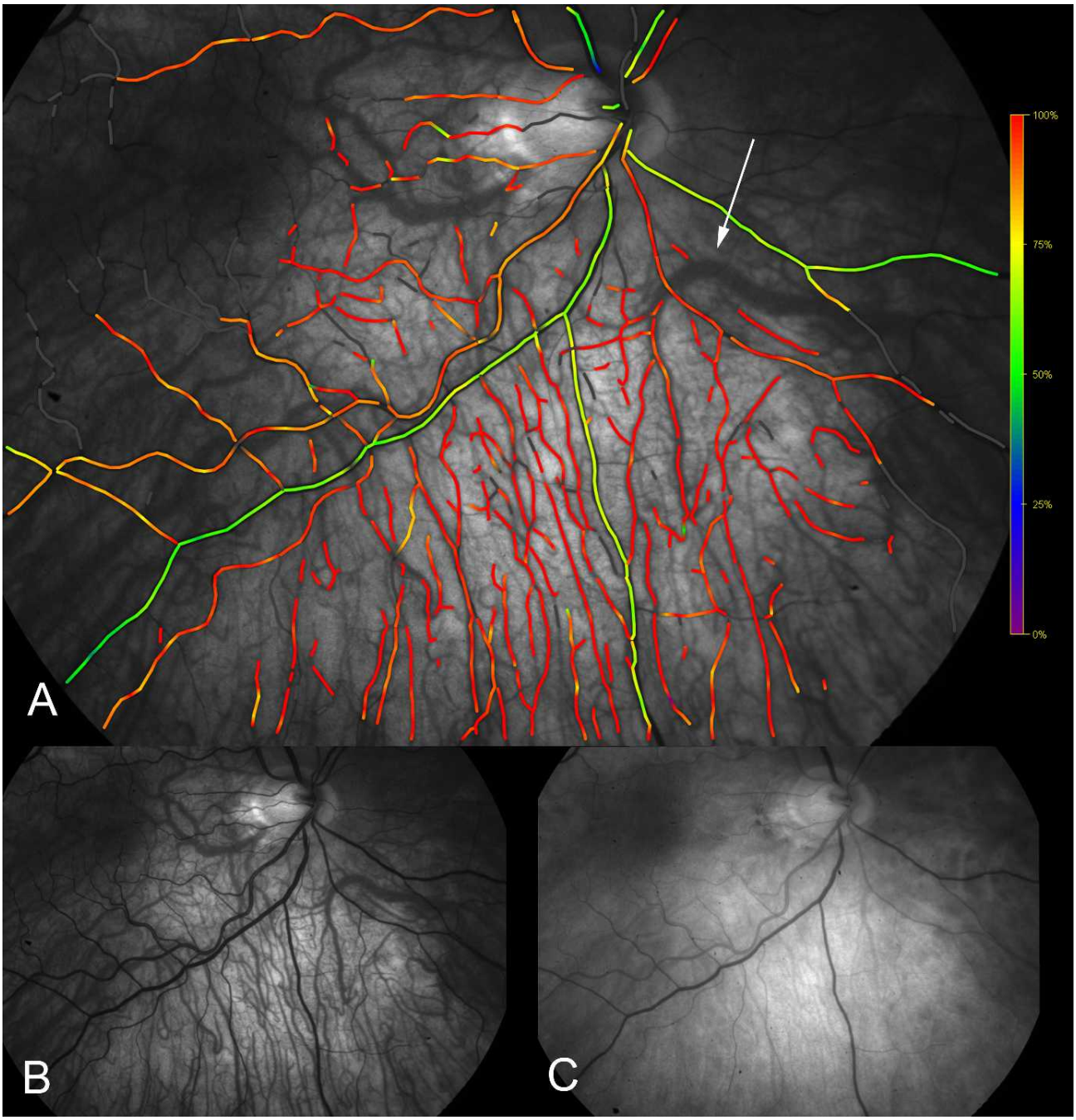

FIGURE 1. Choroidal oximetry image. (**A**) The vascular bed of the choroid is visible and measureable in lightly pigmented individuals with a spectrophotometric oximeter. The oxygen saturation in the vessels is color coded. *Red* represents the highest oxygen saturation and *purple* is the lowest (see *scale* on *right side*). *Arrow* is pointing at a visible choroidal vortex vein. (**B**) Oximetry fundus image taken with oxygen-insensitive wavelength (570 nm). (**C**) The same area of the fundus is captured simultaneously at an oxygen-sensitive wavelength (600 nm).

example, with finger pulse oximeter may be unreliable since the body favors vital organ perfusion including the eyes. Very little is known about the human choroidal oxygen saturation and the effect of disease, and therefore, with further technical development and additional studies, benefits of choroidal oximetry may emerge.

The goal of the study was to establish a new technology to measure hemoglobin oxygen saturation with a spectrophotometric oximeter in the choroidal vasculature in healthy human volunteers.

METHODS

Oxymap T1, a Spectrophotometric Retinal Oximeter

The fundus camera–based oximeter, Oxymap T1 (Oxymap ehf., Reykjavik, Iceland) captures dual-wavelength oximetry images of the retina. The fundus camera is a Topcon TRC-50DX (Topcon Corporation, Tokyo, Japan). The oximeter is attached on top of the fundus camera and is comprised of a custom-

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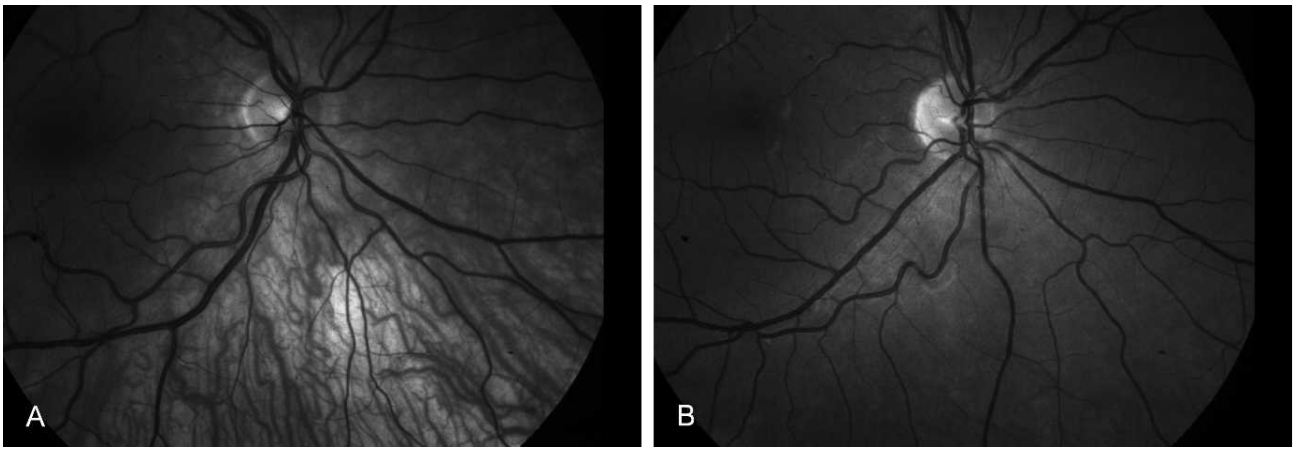

FIGURE 2. Lightly pigmented versus pigmented. Comparison between (**A**) a lightly pigmented individual with visible choroidal vasculature and (**B**) a pigmented individual, choroidal vasculature invisible. The 16 subjects were selected for the study from 148 participants in a normative study solely because their choroidal vasculature was visible and measureable with the retinal oximeter, Oxymap T1.

made optical adapter, image splitter, and two highly sensitive digital cameras (Insight IN1800, 1600 × 1200 square pixels; Diagnostic Instruments Inc., Sterling Heights, MI). The fundus image, captured by the fundus camera (Topcon TRC-50DX; Topcon Corporation), is split into two, and one image is sent to each camera. One camera captures the image at 570 nm (insensitive to oxygen saturation, Fig. 1B), while the other captures the same area of the fundus at 600 nm (sensitive to oxygen saturation, Fig. 1C).

The Oxymap Analyzer (Oxymap Analyzer software 2.2.1, version 3847; Oxymap ehf.) analyzes the images and measures brightness at the measured vessels (I) and to the side of the vessels (I_0). The brightness at the vessel is reduced by light absorbance by the blood in the vessel, while the brightness to the side of the vessel is not. The light absorbance of the blood vessel can be described with the optical density (OD):

$$OD = \log\left(\frac{I_0}{I}\right). \quad (1)$$

The OD at 570 nm is not sensitive to oxygen saturation, whereas the OD at 600 nm decreases with oxygen saturation. The optical density ratio (ODR),

$$ODR = \frac{OD_{600}}{OD_{570}} \quad (2)$$

is therefore sensitive to oxygen saturation, while being relatively insensitive to other effects, such as vessel diameter. The ODR has an inverse and approximately linear relationship to oxygen saturation ($SatO_2$).[16,17]

$$SatO_2 = a + b \times ODR. \quad (3)$$

For further explanation on Oxymap T1 (Oxymap ehf.) and calibration see Geirsdottir et al.[9]

Study Protocol

The study was approved by the National Bioethics Committee of Iceland and the Icelandic Data Protection Authority. All volunteers signed an informed consent before participation in the study. The study adhered to the tenets of the Declaration of Helsinki.

Sixteen subjects were selected from 148 healthy participants from a normative study on retinal oxygen saturation.[9] All subjects were Caucasian, 4 men and 12 women, age of 40 ± 14 years (mean ± SD). The 16 subjects were selected for the study from the 148 participants solely because the choroidal vasculature was visible and measureable with the retinal oximeter (Fig. 1A). Figure 2 shows a lightly pigmented fundus in which choroidal vessels are visible, and for comparison a more densely pigmented fundus, in which these vessels are invisible. Supplementary Figure S1 shows fundus images (570 and 600 nm) from the entire group of 16 eyes. All subjects went through the same standard study and imaging protocol. Five images were acquired from each subject with four different angles of gaze: (1) macula centered, (2) optic disc centered, (3) optic disc down (superior fundus), (4) optic disc up (inferior fundus), and (5) optic disc centered (see Palsson et al.[18]). All images were acquired with the same settings on the fundus camera (Topcon TRC-50DX; Topcon Corporation): 50° field of view, small aperture setting on, and flash intensity 50 W. For further explanation of study and imaging protocol, see Geirsdottir et al.[9]

Analysis

ODRs of choroidal and retinal vessels were obtained using the Oxymap analyzing software (Oxymap Analyzer software 2.2.1, version 3847; Oxymap ehf.). For the normoxia part of the study ($n = 16$) image 4 in the imaging protocol was used for analysis (optic disc up, inferior fundus, Fig. 1A), because the choroidal vessels were most visible in the inferior fundus. The right eye was analyzed in all cases except for two because of bad image quality. ODR was measured for six segments of choroidal vessels (arterioles and venules), one segment of a choroidal vortex vein, one segment of a retinal arteriole, and one segment of a retinal venule. The six choroidal vessel segments were averaged for each subject to get a good average of the area where the choroidal vessels were most visible and also so that the measurement would cover similar area as the retinal arteriole and venule measured for each subject. All measured vessel segments (of all categories) were greater than 50 pixels in length and had a diameter greater than 8.0 pixels (approximately 74 μm[19]). Measurements were not continued beyond branching points. Otherwise, no upper limits were used for length of vessels or diameter. Because most visible choroidal vessels looked alike and it could not be determined whether they are arterioles or venules, they were simply called choroidal vessels. The vortex veins in the choroid were

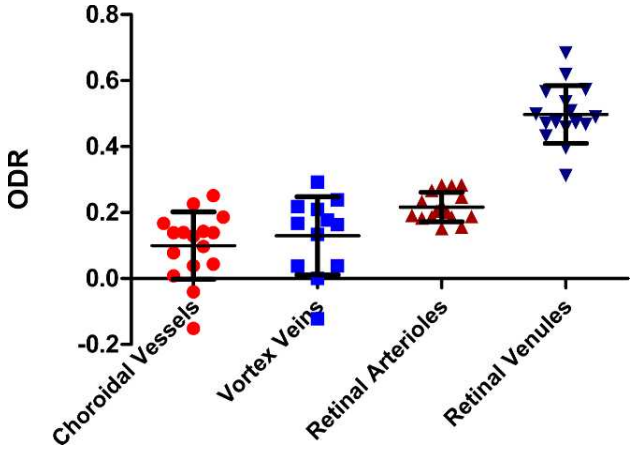

FIGURE 3. ODR during normoxia. Mean ODR, which is inversely related to hemoglobin oxygen saturation, is shown for choroidal vessels, vortex veins, retinal arterioles, and retinal venules under normal oxygen breathing condition (normoxia). The *bars* show one standard deviation. The difference between choroidal vessels and retinal arterioles is statistically significant ($P = 0.0012$, paired *t*-test, $n = 16$). There is no statistically significant difference between choroidal vessels and vortex veins ($P = 0.175$, paired *t*-test, $n = 12$).

recognized by their vortex pattern and wide diameter as indicated with an arrow in Figure 1A. Only 12 of 16 subjects had visible and measurable vortex veins in the inferonasal quadrant of the fundus.

Six of the 16 subjects (two men and four women, age of 42 ± 19 years [mean ± SD]) also inhaled 100% oxygen for 10 minutes (6 L/min, mask covering mouth and nose). Retinal oximetry images were obtained before and immediately after inhalation. Images were analyzed the same way as before except for the choroidal vortex veins, which were not visible in the hyperoxia part of the study because images were acquired with different angle of gaze, optic disk was centered (image 2 in the imaging protocol).

To test the repeatability of the choroidal measurement, the left eye was analyzed using the same criteria as was used for the right eye. Three subjects were excluded, two because of bad image quality and one because the left eye did not have six visible and measureable choroidal segments.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA). A paired *t*-test was applied to compare ODR values for choroidal vessels and retinal arterioles and to compare normoxia and hyperoxia, all categories of measured vessels. For repeatability test a paired *t*-test and one-way ANOVA was applied.

RESULTS

Normoxia

The mean ODR was 0.10 ± 0.10 (mean ± SD) for choroidal vessels, 0.13 ± 0.12 for choroidal vortex veins, 0.22 ± 0.04 for retinal arterioles, and 0.50 ± 0.09 for retinal venules (Fig. 3). According to a paired *t*-test the difference between the choroidal vessels and retinal arterioles was statistically significant ($P = 0.0012$, $n = 16$), but the difference between choroidal vessels and vortex veins was not statistically significant ($P = 0.175$, $n = 12$).

Hyperoxia

Inhalation of 100% oxygen ($n = 6$) lowered ODR levels in choroidal vessels, retinal arterioles, and retinal venules. The decrease in ODR between normoxia and hyperoxia was statistically significant for all vessel types (Fig. 4). The decrease was 0.035 ± 0.028 for choroidal vessels ($P = 0.028$, paired *t*-test), 0.022 ± 0.017 for retinal arterioles ($P = 0.022$, paired *t*-test), and 0.246 ± 0.067 for retinal venules ($P = 0.0003$, paired *t*-test). In addition, the difference between choroidal vessels and retinal arterioles remained statistically significant during hyperoxia ($P = 0.021$, paired *t*-test).

Repeatability

The difference between the right and the left eye was not significant ($P = 0.14$, paired *t*-test). Standard deviation between measurements of the right and left eye in the same individual was 0.07 (Fig. 5).

DISCUSSION

ODR, which correlates inversely to hemoglobin oxygen saturation, can be measured in the choroidal vasculature in lightly pigmented individuals with a spectrophotometric oximeter. Even though calculation of oxygen saturation from choroidal ODR has not been attempted, a low ODR is consistent with high oxygen saturation and high saturation in the choroid is in agreement with earlier studies on oxygenation in animals.[20,21] Shahidi et al.[20] and Shakoor et al.[21] used noninvasive phosphorescence imaging system to measure pO_2 in rat eyes and measured higher pO_2 in the choroid than in retinal arterioles under normal and increased oxygen breathing conditions; this agrees with our findings in the human eye.

While measuring the choroidal vessel ODR is new in this field, measurements on the retinal arterioles and venules is not new and our measurements on the retinal vessels are also in good agreement with previous studies made using an automated image analysis technique based on dual-wavelength oximetry similar to our technique.[7,22]

Alm and Bill[2] found that the arteriovenous difference in oxygen content in the cat choroid is only 3%. This agrees with our experience that choroidal arterioles and venules are difficult to distinguish with spectrophotometric oximetry and that choroidal vortex veins only have slightly higher ODR (indication of lower oxygen saturation) than the other choroidal vessels (Fig. 3).

The Oxymap T1 oximeter (Oxymap ehf., Reykjavik, Iceland) has been shown to be sensitive to changes in oxygen saturation in retinal vessels and to give repeatable and reliable results when measuring hemoglobin oxygen saturation and vessel diameter.[8,9,18,19] This is the first time it has been applied to choroidal vessels. By using the standard Oxymap T1 retinal calibration for the choroidal ODR the calculated hemoglobin oxygen saturation is 107 ± 12% (mean ± SD, $n = 16$) for the choroidal vessels, 106 ± 13% for the vortex veins (mean ± SD, $n = 12$), 94 ± 5% for retinal arterioles (mean ± SD, $n = 16$), and 59 ± 9% (mean ± SD, $n = 16$) for retinal venules. This calibration is obviously not appropriate for the vessels within the choroid, but the oxygen saturation for the retinal vessels compares well to our previous results on retinal oxygen saturation.[8,9] Subjects for this study were selected because their choroidal vasculature was visible (due to light pigmentation) and measureable. That does not seem to affect the results on oxygen saturation for the retinal arterioles and venules. Inhalation of 100% oxygen (hyperoxia, $n = 6$) lowered the ODR levels for all measured vessel types, both choroidal and retinal, which corresponds to an increase in oxygen

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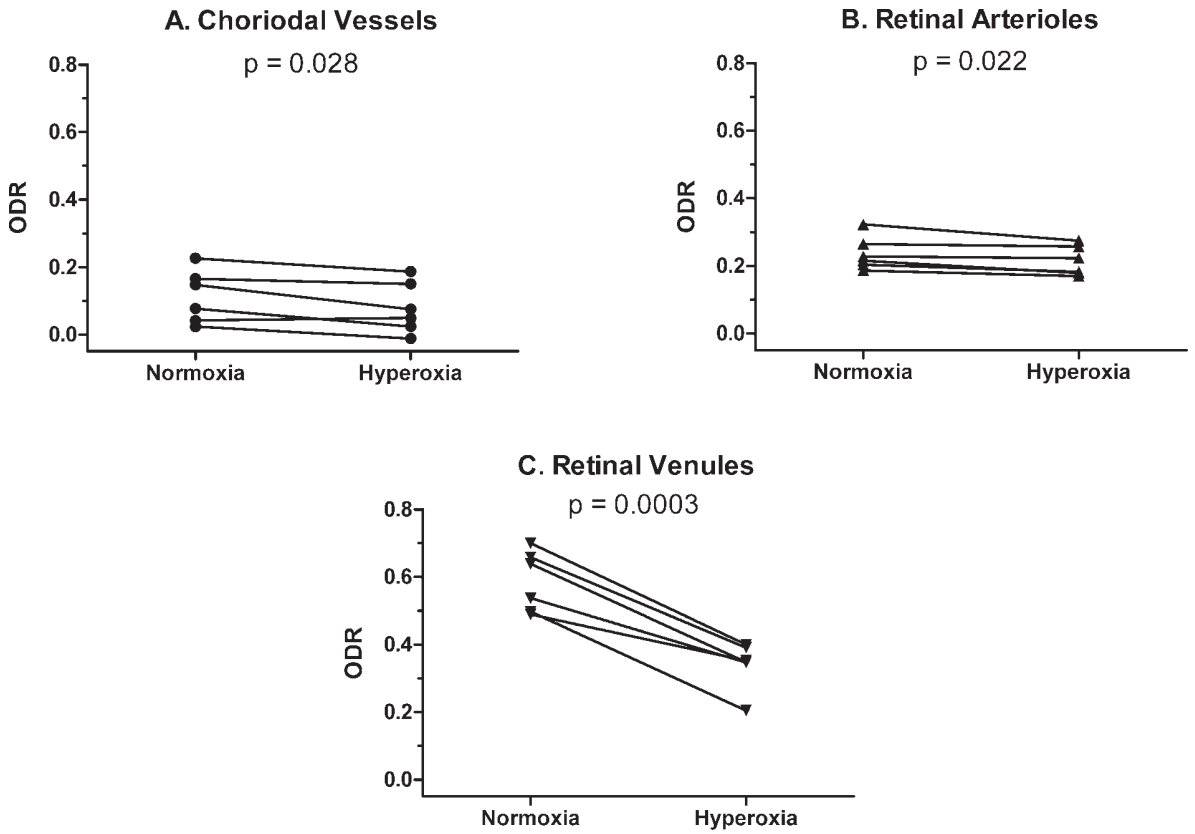

FIGURE 4. ODR during normoxia and hyperoxia. ODR is inversely related to hemoglobin oxygen saturation. It is shown for (**A**) choroidal vessels, (**B**) retinal arterioles, and (**C**) retinal venules under normal oxygen breathing condition (normoxia) and with subjects breathing 100% oxygen (hyperoxia). According to paired *t*-test there is a statistically significant difference for choroidal vessels ($P = 0.028$), retinal arterioles ($P = 0.022$), and retinal venules ($P = 0.0003$) between normoxia and hyperoxia.

saturation. By using the standard retinal calibration, the increase in hemoglobin percentage was found to be 4% for choroidal vessels, 2% for retinal arterioles, and 26% for the retinal venules. This demonstrates that with hyperoxia the oximeter is sensitive to changes in oxygen saturation for both the choroidal and retinal vessels. (Vortex veins were not visible on images taken for the hyperoxia part of the study.)

We measured only individuals with the most visible choroidal vessels, which included only 16 individuals from a group of 148 healthy individuals. Of these, only six were available for the hyperoxia experiment. The small sample sizes may make the parametric statistical tests used vulnerable to deviations of the population from normal distribution. We therefore recalculated all *P* values using the Wilcoxon signed rank test. This did not change the conclusions of the study, although the difference between normoxia and hyperoxia in choroidal vessels became borderline statistically significant ($P = 0.063$), and the same was true for the comparison of retinal arterioles and choroidal vessels during hyperoxia ($P = 0.059$).

The repeatability of retinal oxygen measurements with the oximeter has been determined previously.[18] The standard deviation of repeated measurements was 1.0% for arterioles and 1.4% for venules. Repeatability was not tested in the same way in this study but can nonetheless be estimated. We calculated the standard deviation between measurements of the left and right eye in the same individual. This is displayed in ODR values in the results. If the ODR values are transformed into saturation (with standard retinal calibration), the standard deviation between measurements of the left and right eye in the same individual was 7% (Fig. 5). Although the study was not designed to estimate repeatability, these values indicate that the variability is greater for the choroidal measurements than it is for retinal measurements.

The optical properties of the intravascular tissue in the choroid may play a role in these measurements. The ODR was lower in the choroidal vessels than in the retinal arterioles,

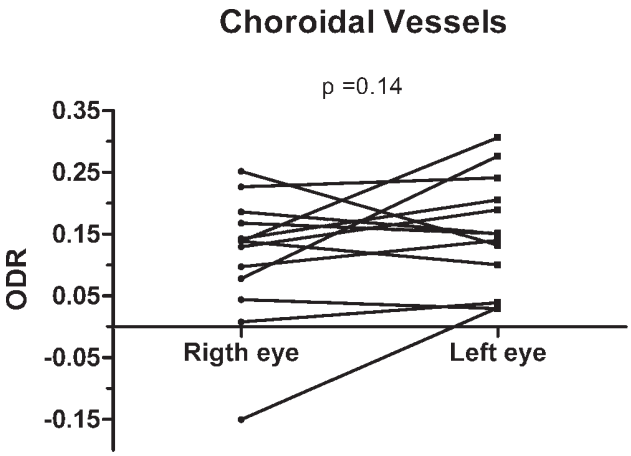

FIGURE 5. Repeatability: right versus left eye. For repeatability testing the left eye was measured using the same criteria as was used for the right eye. According to a paired *t*-test the difference was not significant ($P = 0.14$). Standard deviation between measurements of the right and left eye in the same individual was 0.07 ODR values.

potentially indicating higher oxygen saturation in the choroid; however, it is also observed that the OD of choroidal vessels is reduced at both 570 and 600 nm, which can affect the ODR and oxygen saturation conversion. This is compatible with scattering of light within the choroid reducing the contrast of choroidal vessels as follows. The OD of retinal vessels is measured against a bright background dominated by scattering from interstitial tissue. Choroidal blood vessels are embedded within this interstitial tissue and components of this tissue lying between blood vessels and the retinal pigment epithelium backscatter incident illumination, which reduces contrast of choroidal vessels; that is, it reduces OD. The magnitude of the scattering from interstitial tissue is approximately equal at both 570 and 600 nm but causes a proportionately greater reduction in the OD for the lower OD measurements at 600 nm. In consequence, there is a reduction in the ODR for choroidal vessels. If the reduction in ODR due to scattering is neglected, this would imply higher oxygenation levels and suggests nonrealistic oxygen saturation in excess of 100% in choroidal vessels. It is probable that the different illumination of the vessel by the surrounding choroidal tissue also has an effect, though this is expected to be less significant.

Different optical properties result from the fact that retinal vessels and choroidal vessels lie in different tissues at different depths. The result is that the standard calibration, which has been used for retinal vessels to transform ODR into oxygen saturation, is not appropriate for choroidal vessels. However, the lowering of ODR in the choroidal vessels with hyperoxia demonstrates that the oximeter is sensitive to changes in oxygen saturation in choroidal vessels as well as in retinal vessels.

It is furthermore observed that for some of the imaged retinas, the OD of choroidal vessels imaged at 600 nm is negative; that is, vessels appear brighter than the surrounding choroidal tissue, and this leads to the negative ODRs shown in Figure 3. Study of these images suggests that this brightness is associated with diffuse structure in the scattering interstitial tissue that correlates with the vessel structure and that there is insufficient contrast to detect an OD due to the vessel. This may be because the vessels are located more deeply within the choroidal tissue than the vessels for which positive ODs can be measured. In these cases the ODR for these vessels is effectively zero.

The physical optics leading to the observed ODR of choroidal vessels is inherently different from that underpinning oximetry of retinal vessels. The determination of choroidal vessel oxygenation will require some modification and refinement to the physical optics model established for retinal vessel oximetry, and this is the subject of ongoing investigation. It is clear, however, from these results that it is nevertheless possible to detect changes in choroidal vessel oxygenation associated with changes in inspired oxygen.

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Appendix B

Retinal Oximetry with a Scanning Laser Ophthalmoscope

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Key words: hyperoxia, hypoxia, oxygen, hemoglobin, retina
Word count: 2.204 words

Abstract

Purpose:

The purpose of the study was to assess if a scanning laser ophthalmoscope (SLO), Optomap 200Tx, could be used for measurements of hemoglobin oxygen saturation in retinal blood vessels in healthy volunteers.

Methods:

Optomap 200Tx, uses two lasers for image acquisition, 532 and 633 nm. Retinal images of healthy individuals and patients with retinal vein occlusion were analyzed with modified Oxymap Analyzer software, which tracks retinal vessels and calculates relative hemoglobin oxygen saturation.

Results:

Oxygen saturation in healthy individuals was measured as 92%±13% for arterioles and 57%±12% for venules (mean±SD, n=11, p=0.0001). Standard deviation for repeated measurements of the same eye was 3.5 percentage points for arterioles and 4.4 percentage points for venules. In patients with confirmed venular hypoxia, central retinal vein occlusion (CRVO) or hemivein occlusion, the venular oxygen saturation was 23%±3% in the affected eye and 59%±3% in the fellow eye (n=4, p=0.0009).

Conclusions:

It is technically possible to derive information on retinal oxygen saturation from an SLO with two wavelength oximetry algorithm. The system produced both sensitive and repeatable results. The remaining challenges include decreasing variability between vessels of the same eye and variability between individuals. Given the advantages that SLO imaging has over conventional fundus camera optics in retinal oximetry, further development of SLO oximetry may provide the optimal approach to retinal oximetry.

Introduction

Measurement of hemoglobin oxygen saturation in blood vessels is based on different light absorption of oxyhemoglobin and deoxyhemoglobin. Current conventional approach to retinal oximetry consists of white light illumination of the fundus where the image is subsequently filtered with light filters for spectrophotometric analysis. Two such retinal oximetry systems are currently available commercially (Oxymap ehf., Reykjavík, Iceland and Imedos, Jena, Germany).[1, 2] Both use conventional fundus camera optics and dual wavelength analysis. Other researchers in retinal oximetry have used various optical approaches with either two wavelengths or multispectral analysis.[3-9]

Scanning laser ophthalmoscope (SLO) can also be used to measure retinal hemoglobin oxygen saturation. Studies on SLO oximetry have been published mainly by three other research groups, Smith and Denninghoff et al., Ashman et al. and Li et al.[10-12] In 1998, Smith et al. reported on a two-wavelength scanning laser Eye Oximeter (EOX) prototype, which was used for retinal oxygen saturation measurements in anesthetized swine during blood loss. Strong correlation was found between retinal arterial and femoral arterial oxygen saturation, and retinal venous saturation and blood loss.[12] In 1999, this same research group reported on a second generation of the EOX for studies on retinal oxygen saturation in human subjects. The EOX was mounted on a slit lamp base and four diode lasers at wavelengths 629, 678, 821 and 899 nm used to image the retina. Oxygen saturation was found to be 65% and 101-102% in the retinal arteries and veins, respectively, suggesting that the EOX is sensitive to retinal oxygen saturation.[13] In 2011, Denninghoff et al. reported on the use of a new modified confocal SLO (ROx-3) for blue-green oximetry (BGO) in both swine and human subjects. Results on retinal oxygen saturation were in the same range as reported by other research groups.[14] Ashman et al. used a prototype SLO to measure the retinal oxygen saturation under different percentages of oxygen breathing mixtures; 10%, room air and 100%. Lasers used for

imaging were 633 nm and 815 nm. A difference was seen between retinal arterioles and venules but no difference was measureable between different oxygen breathing mixtures.[10] Li et al. measured the oxygen saturation in small retinal vessels (diameter<50µm) using adaptive optics confocal SLO with two wavelengths, 680 and 796 nm. A difference between arterioles and venules was detected.[11]

SLO offers some advantages for retinal oximetry. It uses lasers to create monochromatic images at two or more wavelengths which minimizes unnecessary light exposure to the fundus. SLO can easily be used with un-dilated pupils and penetrates cataracts and other optical opacities in the eye better than conventional spectrophotometric fundus camera. It also allows wide field scanning of almost the entire fundus whereas conventional fundus cameras are limited to relatively narrow images of the posterior pole. Given these advantages of SLO over the conventional approach we set out to develop a system for retinal SLO oximetry. We combined the use of SLO, Opomap 200Tx, developed by Optos plc. (Dunfermline, Scotland, UK) and oximetry image analysis software developed by Oxymap ehf. The system was developed and tested in healthy human subjects breathing either room air or pure oxygen and in patients with confirmed retinal venous hypoxia.

Methods

Study protocol

The study was approved by the National Bioethics Committee of Iceland and the Icelandic Data Protection Authority. All participants signed an informed consent before participation in the study. The study adhered to the tenets of the Declaration of Helsinki.

Scanning laser oximetry

The SLO, Optomap 200Tx (Optos) uses two lasers, 532 nm (green) and 633 nm (red) to capture fundus images. The images from the Optomap 200Tx can, in principle, be used for

dual wavelength retinal oximetry; the 633 nm image is sensitive to hemoglobin oxygen saturation while the 532 nm image is close to being insensitive and can be used as a reference. The Oxymap Analyzer software (Oxymap ehf.) processes the two spectral images. The software tracks retinal vessels and estimates optical density (OD) at each point along the vessels at each wavelength and calculates the optical density ratios (ODRs), which are inversely related to oxygen saturation. The ODs and ODRs are calculated according to following equations:

$$OD = log\frac{I_0}{I} \qquad (eq.1)$$

$$ODR = \frac{OD_{633}}{OD_{532}} \qquad (eq.2)$$

were I is the light intensity inside the retinal vessel and I_0 is the light intensity outside the vessel. After calibration, the oxygen saturation ($SatO_2$) can be calculated according to following:

$$SatO_2 = a + b \cdot ODR,$$

The constants, a and b, were found by matching the average arteriolar and venular ODRs from the healthy subjects with previously published mean retinal oxygen saturation values in healthy individuals, obtained with a calibrated device by Schweitzer et al.[15] The previous study found that retinal arteriolar saturation was 92.2% and venular saturation was 57.9%. Matching resulted in following values: a=-2.4733 and b=1.4388.

The vessel detection algorithm of Oxymap Analyzer software was modified in order to process the larger images (100°) of the SLO and take into account the different magnification compared with conventional Oxymap T1 fundus camera based system (50°). For further explanation of the Oxymap T1 system and Oxymap Analyzer software, see Geirsdottir et al.[1]

Feasibility and repeatability

For initial adaption and testing of the Optomap 200Tx SLO as a retinal oximeter, two 100° (ResMax setting) fundus images were acquired from 11 healthy volunteers (6 males and 5 females), age 34±10 years (mean±SD). Images were acquired of an un-dilated right eye of each subject. The Optomap 200Tx was set to store un-scaled 12 bit images and red and green sensors set at gain 2 (for lightly pigmented iris). The first image from each subject was used for initial assessment of the device as a retinal oximeter, to test if there was a clear difference between arterioles and venules. Two images were acquired of the right eye of each subject to test the repeatability of measurements between images.

Sensitivity

Two different methods were used to investigate the sensitivity of the device, pure oxygen inhalation and measurements of patients with confirmed retinal venous hypoxia due to either central retinal vein occlusion (CRVO) or hemivein occlusion:

1. Two healthy subjects were measured before and after inhalation of pure oxygen for 10 minutes (10L/min), mask covering both mouth and nose. Images were acquired before oxygen inhalation started (baseline image), when oxygen inhalation ended and every 5 seconds for the next 135 seconds during recovery and then finally after 10 minutes of recovery.
2. Three patients with CRVO and one patient with hemivein occlusion were also measured. Hypoxia had been confirmed with the Oxymap T1 oximeter.[16] CRVO affected eye was compared to the fellow eye in the same patient. For the patient with hemivein occlusion the affected area (inferior fundus) was compared to the same area in the fellow eye.

Image analysis

All oximetry images were analyzed in a standardized manner. Normoxia and hyperoxia images were all analyzed in the same way; oxygen saturation was measured in the main superotemporal vessel pair (retinal arteriole and venule). For CRVO patients, all major retinal arterioles and venules above 6 pixels in diameter were measured (6 pixels in are approximately 60 μm). Retinal vessels with diameter smaller than that are excluded from analysis because the saturation measurement can be unreliable.

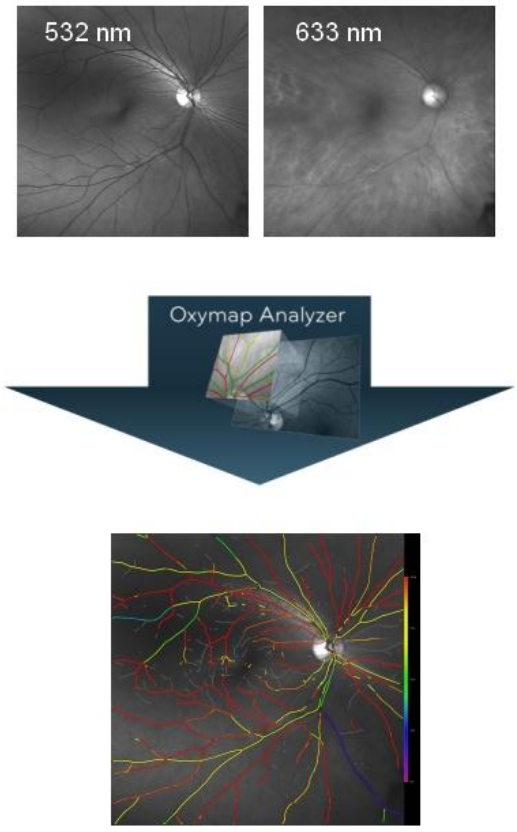


Figure 1.Oximetry fundus image of a healthy retina. The scanning laser ophthalmoscope (SLO), Optomap 200Tx, uses two lasers to acquire fundus images, 532 nm (green) and 633 nm (red). The software, Oxymap Analyzer, processes the two spectral images and calculates hemoglobin oxygen saturation of the retinal blood vessels (see bottom image). Red color represents 100% oxygen saturation and violet color 0% oxygen saturation (see scale on right side).

Statistical Analysis

Statistical analysis was performed using Graphpad Prism 5.03 (GraphPad Software Inc., LaJolla, CA, USA). A paired t-test was applied to test the difference between arterioles and venules and between affected eyes and healthy fellow eyes for CRVO/hemivein occlusion patients. Standard deviation between two repeated measurements of the same vessel was calculated using the square root of the within subjects mean square from ANOVA as recommended by Bland.[17]

Results

Oxygen saturation was measured as 92%±13% (n=11, mean±SD) for arterioles and 57%±12% for venules (the mean saturation values are a direct result of calibration). The difference between arterioles and venules was statistically significant according to a paired t-test (p=0.0001). Standard deviation for repeated measurements of the same vessel was 3.5 percentage points for arterioles and 4.4 percentage points for venules. Figure 2 highlights the difference between arteriolar and venular saturation in each individual. Figure 3 shows the repeatability while figure 4 shows results of oxygen breathing experiments for test of sensitivity. Figure 5 shows an example of how the pseudo color map of retinal oxygen saturation changes with breathing of pure oxygen. The table and figures 6 and 7 show the results of measurements on patients with CRVO or hemivein occlusion.

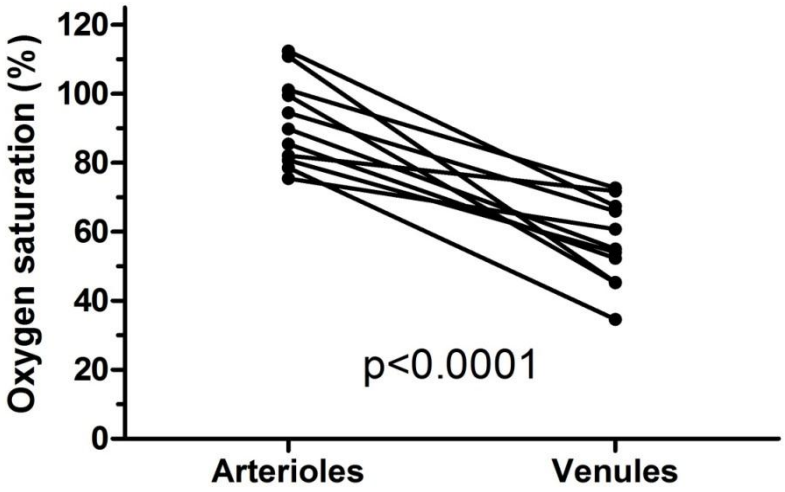


Figure 2. The graph shows the oxygen saturation of the main superotemporal vessel pair (arteriole and venule) for 11 healthy subjects. The lines connect vessels in the same eye. According to a paired t-test the difference between arterioles and venules is statistically significant (p<0.0001).

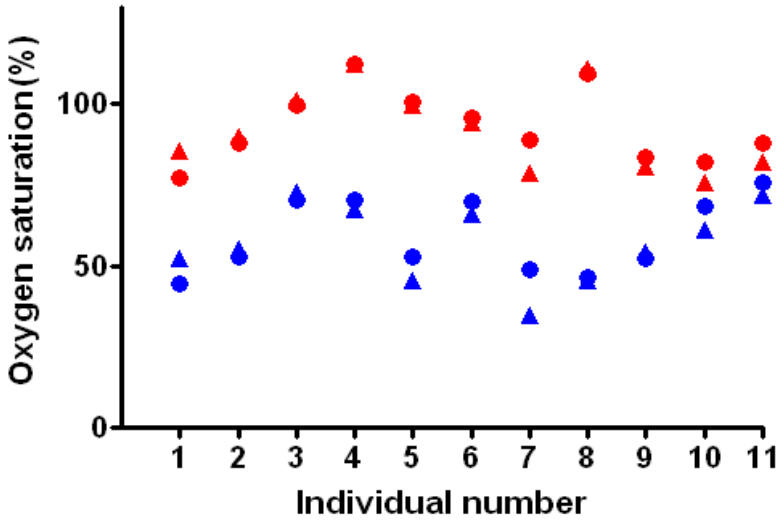


Figure 3. The graph shows oxygen saturation of the main superotemporal vessel pair (arteriole and venule) from 11 healthy subjects. Two images were analyzed for each subject. The triangle denotes the first image for each individual and the circle denotes the second image. (Red=arterioles and blue=venules).

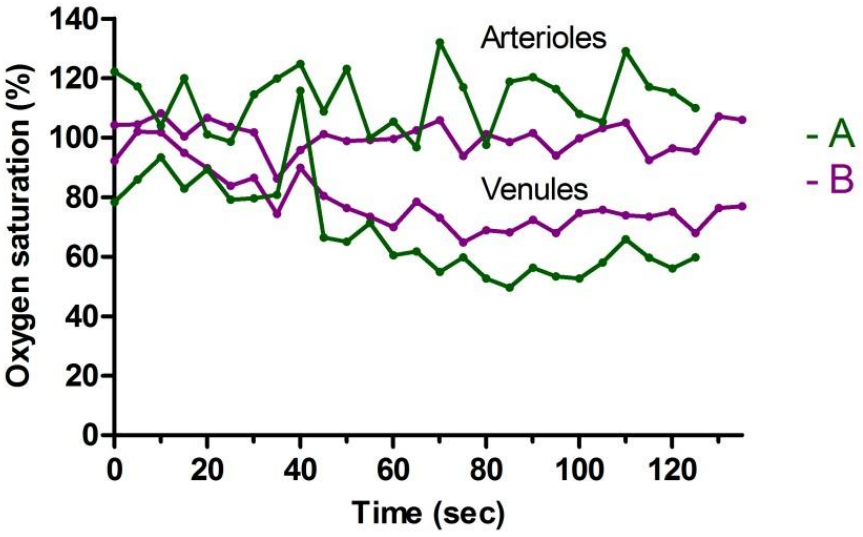


Figure 4. The figure shows oxygen saturation over time for retinal arterioles and venules for two healthy subjects (A and B). Subjects inhaled pure oxygen for 10 minutes (10L/min) and fundus images were acquired with the Optomap 200Tx after the inhalation ended (time=0) and every 5 seconds for 135 seconds during breathing of room air. Oxygen saturation was analyzed using Oxymap Analyzer software.

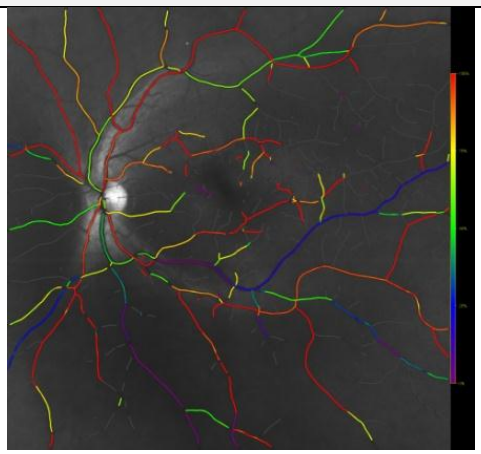
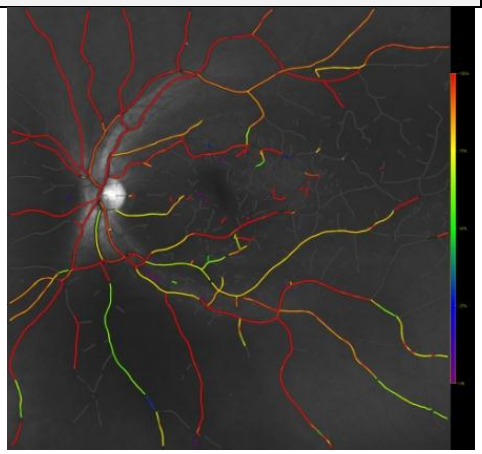
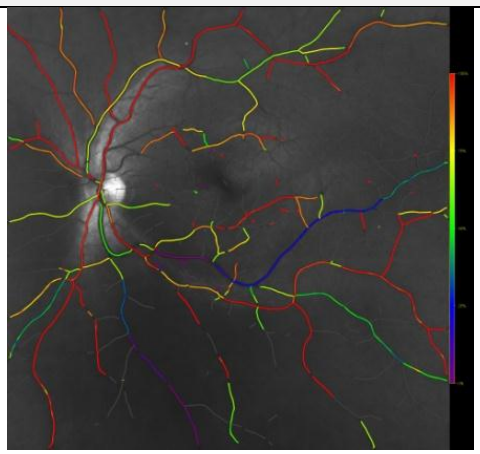
Before inhalation (baseline)	After 10 min. inhalation of pure O_2 (time = 0 min)	After 10 min. recovery with room air (time = 10 min)
		

Figure 5. Oximetry fundus images from one healthy subject acquired with the scanning laser ophthalmoscope (SLO), Optomap 200Tx and analyzed with Oxymap Analyzer software before and at different time after inhalation of pure oxygen.

Table 1. The table shows oxygen saturation (mean±standard deviation) of four patients with either central retinal vein occlusion (CRVO) (pat.1-3) or hemivein occlusion (pat. 4). The affected eye is compared to healthy fellow eye. Difference between retinal arterioles in affected eyes and fellow eyes was not statistically significant (n=4, p=0.6405, paired t-test), difference between retinal venules in affected eyes and fellow eyes was statistically significant (n=4, p=0.0009, paired t-test)

Oxygen saturation (%) in retinal vessels (mean ± standard deviation)				
	Affected eye		Fellow eye	
Pat.no.	Arterioles	Venules	Arterioles	Venules
1	109 ± 21	25 ± 42	110 ± 13	56 ± 5
2	108 ± 9	26 ± 26	101 ± 14	60 ± 22
3	115 ± 11	19 ± 13	116 ± 8	59 ± 24
4	95 ± 10	20 ± 28	109 ± 15	63 ± 7
Mean	107 ± 9	23 ± 3	109 ± 6	59 ± 3

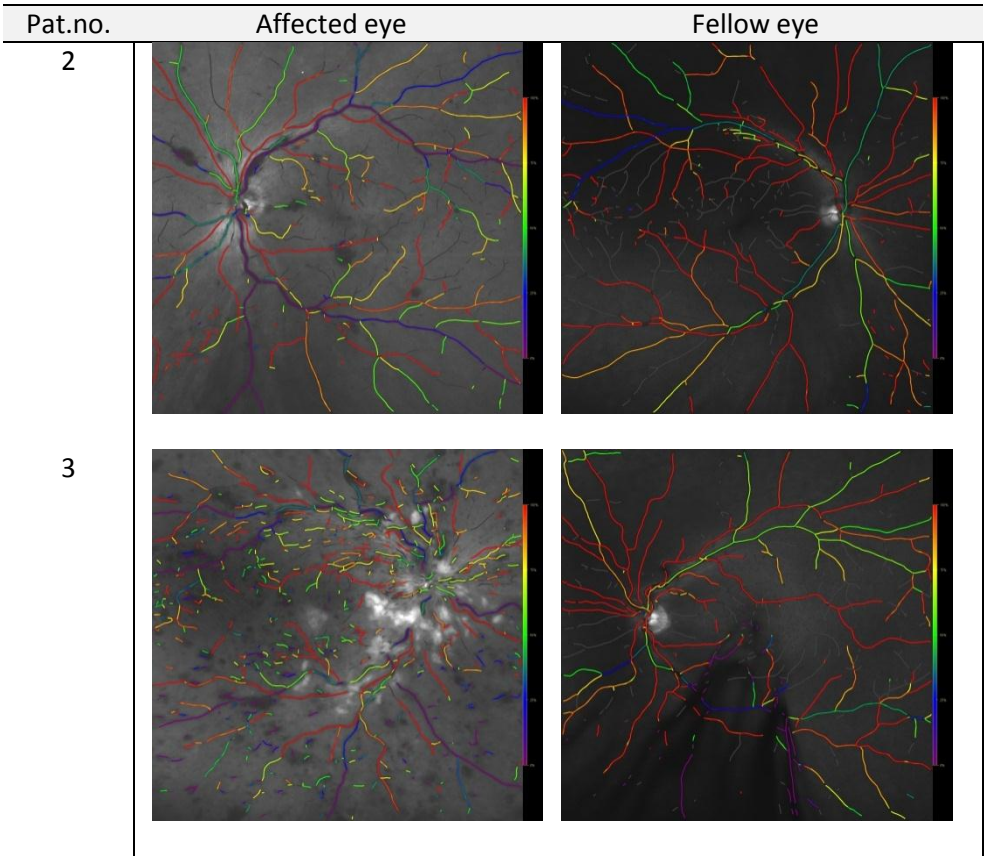


Figure 6. Oximetry fundus images from two patients with CRVO (patients nr. 2 and 3, see table 1), acquired with Optomap 200Tx and analyzed with Oxymap Analyzer software. A clear difference is seen in venular oxygen saturation between the CRVO affected eye and the fellow eye (blue color denotes approximately 25% oxygen saturation, see scale on right side).

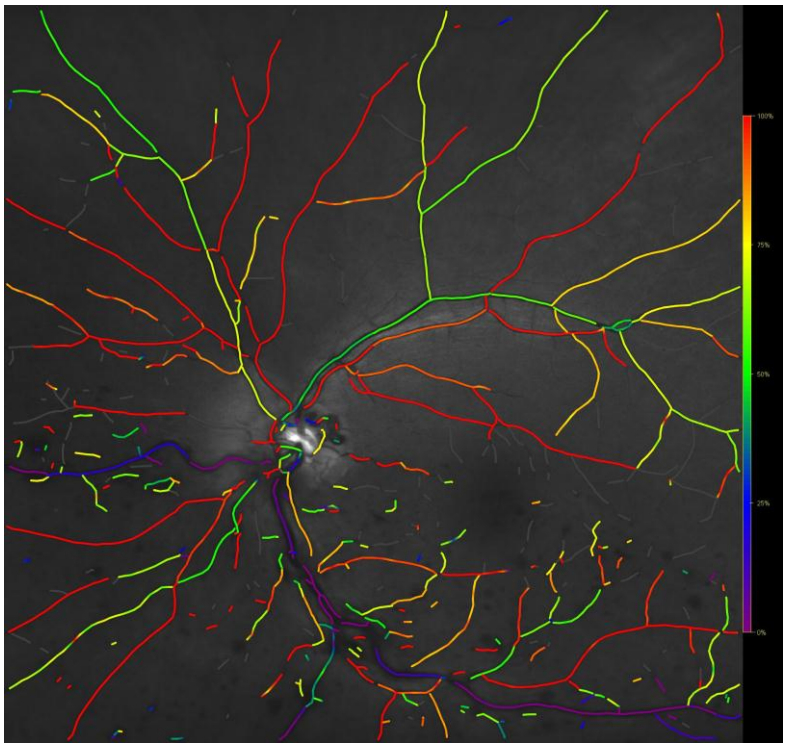


Figure 7 Oximetry fundus image from an individual with hemivein occlusion. Images acquired with Optomap 200Tx and analyzed with Oxymap Analyzer software. A clear difference is seen in oxygen saturation between the superior and inferior retinal venules, inferior venules are occluded.

Discussion

The combination of Optomap 200Tx SLO imaging and Oxymap Analyzer software was successful in the development of an SLO based retinal oximetry system. SLO oximetry provided oxygen saturation measurements in retinal arterioles and venules and the difference between arterioles and venules is statistically significant, which is the first confirmation that the technique is working.

The repeatability of the measurement is good as indicated by the standard deviation for repeated measures. It is 3.5% for arterioles and 4.4% for venules, which is acceptable considering the early stage in the development. The more developed fundus camera based Oxymap T1 has standard deviation for repeated measures of 1.0% for arterioles and 1.4% for venules.[18]

The main difficulty is the considerable variability between vessels of the same eye. An example of this can be seen in the healthy eye in figure 5 (baseline and time=10 min), where several vessel segments show abnormally low saturation. The variability between individuals is also considerable as evidenced by the standard deviations for the group of 11 healthy individuals, 13% for arterioles and 12% venules and as seen in figure 3. Even though the variability is greater than seen with Oxymap T1 retinal oximetry system today, it is similar to the variability seen in the early days of developing the Oxymap system. Technical optimization, both hardware and software, proved successful in reducing the variability of oxygen saturation measurements with the Oxymap system. Similar technical improvements are needed for the SLO oximetry system to reduce the intra-and inter subject variability.

The sensitivity of the SLO oximetry system to different oxygen saturations can be seen both in the different measurements in arterioles and venules as well as the response to breathing either room air or 100% oxygen which also demonstrates the ability to detect changes in oxygen saturation over time. In CRVO, SLO oximetry clearly detects the difference between the oxygen saturation in CRVO affected eyes and the healthy fellow eyes and provides picturesque wide field images of the variable hypoxia in CRVO or hemivein occlusion.

In the current study, the 100° ResMax image was used for SLO oximetry. However, retinal oximetry images could also be produced with the 200° wide field SLO imaging but this would require further software modification. Further development of the wide field imaging for oximetry would allow oximetry analysis of the peripheral fundus, which could be useful in various eye diseases e.g. diabetic retinopathy.

The SLO oximetry system has also been used to image patients with a variety of other eye diseases including diabetic retinopathy, age related macular degeneration and retinal detachment and the use of SLO oximetry in these diseases will be analyzed further in future

studies. The SLO oximetry images were generally acquired with un-dilated pupils and the system was able to acquire useful images in eyes with cataracts and other ocular opacities, where conventional fundus camera based oximetry was more difficult.

In summary, the combination of SLO imaging and retinal oximetry software was successful in developing a system for SLO oximetry which is both sensitive and gives repeatable results. The intra- and inter-subject variability is still large and further hardware and software development is needed. Given the advantages that SLO imaging has over conventional spectrophotometric fundus cameras in retinal oximetry, further development of SLO oximetry may provide the optimal approach for retinal oximetry.

Acknowledgement

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