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Retinal hemodynamic oxygen reactivity assessed by perfusion velocity, blood oximetry and vessel diameter measurements

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ABSTRACT.

Purpose: **To test the oxygen reactivity of a fundus photographic method of measuring macular perfusion velocity and to integrate macular perfusion velocities with measurements of retinal vessel diameters and blood oxygen saturation.**

Methods: **Sixteen eyes in 16 healthy volunteers were studied at two examination sessions using motion-contrast velocimetry and retinal oximetry with vessel diameter corrections. To test oxygen reactivity, participants were examined during normoxia, after 15 min of hyperoxia and finally after 45 min of normoxia. Repeatability was assessed by intraclass correlation coefficients (ICC) and limits of agreement.**

Results: **Fifteen minutes of hyperoxia was accompanied by mean reductions in arterial and venous perfusion velocities of 14% and 16%, respectively ($p = 0.0080$; $p = 0.0019$), constriction of major arteries and veins by 5.5% and 8.2%, respectively ($p < 0.0001$), increased retinal arterial oxygen saturation from 95.1 ± 5.0% to 96.6 ± 6.4% ($p = 0.038$) and increased retinal venous oxygen saturation from 62.9 ± 6.7% to 70.3 ± 7.8% ($p = 0.0010$). Parameters returned to baseline levels after subsequent normoxia. Saturation and vessel diameter ICCs were 0.88–0.98 (range). For perfusion velocities, short-term ICCs were 0.79–0.82 and long-term ICCs were 0.06–0.11. Intersession increases in blood glucose were associated with reductions in perfusion velocities (arterial $p = 0.0067$; venous $p = 0.018$).**

Conclusion: **Oxygen reactivity testing supported that motion-contrast velocimetry is a valid method for assessing macular perfusion. Results were consistent with previous observations of hyperoxic blood flow reduction using blue field entoptic and laser Doppler velocimetry. Retinal perfusion seemed to be regulated around individual set points according to blood glucose levels. Multimodal measurements may provide comprehensive information about retinal metabolism.**

Key words: glucose – hyperoxia – oximetry – oxygen – retina – retinal blood flow – vessel diameters

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Introduction

Blood flow through the retinal circulation is subject to blood pressure autoregulation and regulation in response to the concentrations of nutrients, metabolites and blood gases (Friedman & Smith 1965; Pournaras et al. 2008; Kur et al. 2012). Presumably, these responses reflect retinal metabolic demand. Retinal vascular function can be investigated by non-invasive fundus photographic methods which were used in this study to assess three markers of retinal vascular function in healthy volunteers, namely retinal trunk vessel diameters and oxygen saturations and macular perfusion velocities. These markers were chosen because they have contributed to the understanding of retinal pathophysiology and may offer an opportunity for early detection of pathological conditions, for example, diabetic retinopathy (Feke et al. 1994; Gilmore et al. 2007a; Justesen et al. 2010; Kur et al. 2012; Jorgensen et al. 2014). Studies have mainly included single measurement modalities. It seems likely that concomitant registration of multiple measures of retinal vascular characteristics could provide a more comprehensive overview of retinal vascular function and, indirectly, of retinal metabolism. Macular perfusion velocity can be measured using a relatively new technique based on motion-contrast imaging which has demonstrated abnormal blood flow in patients with retinal disease (Burgansky-Eliash

et al. 2010, 2014; Beutelspacher et al. 2011) and has previously been validated in vitro (U.S.Food & Drug Administration 2008). The primary purpose of this study was to examine retinal responses to transient hyperoxia and to test the validity of motion-contrast velocimetry in vivo in man. A secondary purpose was to integrate macular perfusion velocities with measurements of retinal vessel diameters and blood oxygen saturation. Specifically, we aimed to characterize associations between the outcome variables and to identify possible confounders by testing for associations with anthropometric and biochemical characteristics. Acute hyperoxia was chosen as a test paradigm because it has been shown to reduce retinal perfusion, increase retinal oxygen saturation and constrict retinal vessels (Hickam & Frayser 1966; Gilmore et al. 2005; Hardarson et al. 2006; Pournaras et al. 2008), thus allowing concomitant changes in all three vascular parameters. The oxygen reactivity of the macular microcirculation as measured by blue field entoptic and laser Doppler velocimetry was used as historical references (Sponsel et al. 1992; Strenn et al. 1997; Kiss et al. 2002; Tomic et al. 2005).

Table 1. Characteristics of healthy participants.

	Visit 1	Visit 2
Number	16	
Female/Male	12/4	
Age	24 (19–55)	
Spherical equivalent refraction (*D*)	−1.87 ± 2.27	
Visual acuity (ETDRS letters)	87 (84–96)	
Intraocular pressure (mmHg)	13.5 (11–20)	13.5 (10–19)
Axial length (mm)	24.54 ± 1.33	
Height (cm)	174.1 ± 8.7	
Weight (kg)	73.4 ± 13.6	73.2 ± 12.7
BMI (kg/m^2)	24.1 ± 3.5	24.0 ± 3.1
Systolic blood pressure (mmHg)	130 (91–139)	124 (106–134)
Diastolic blood pressure (mmHg)	81 (54–92)	79 (64–89)*
Mean arterial pressure (mmHg)	97 (66–107)	94 (82–104)*
Heart rate (beats/min)	62 (47–92)	62 (49–94)
Average retinal thickness (μm)†	280.1 ± 7.9	
Central subfield thickness (μm)†	262.3 ± 23.5	
Retina nerve fibre layer thickness (μm)†	89.1 ± 8.8	
Blood haemoglobin (mmol/l)	8.8 ± 0.7	8.4 ± 0.7*
Blood glucose (mmol/l)	4.4 ± 0.8	4.4 ± 0.7
Plasma cholesterol (mmol/l)	5.1 ± 1.1	4.7 ± 0.6
Blood haemoglobin A1c (mmol/mol)	32.4 ± 2.4	32.2 ± 2.1
Plasma creatinine (μmol/l)	70.6 ± 7.8	71.3 ± 9.4
Plasma sodium (mmol/l)	143.5 ± 1.7	142.8 ± 1.6
Plasma potassium (mmol/l)	4.1 ± 0.3	4.2 ± 0.4

Results are reported as mean ± SD or median (range). The oxygen reactivity test was performed at visit 2.
*$p < 0.05$ for comparison between the two visits.
†Measurements derived from spectral-domain optical coherence tomography (Cirrus SD-OCT).

Materials and Methods

Participants

The study included 16 healthy, non-smoking volunteers (four men and 12 women) with a median age of 24 years (range 19–55 years; Table 1). Exclusion criteria were any clinically significant systemic conditions, ocular disease, large refractive errors (spherical equivalent of more than +/− 6 dioptres and/or astigmatism of more than +/− 3 dioptres) and suboptimal cooperation for photographic procedures. Use of systemic medications other than oral contraception or hormone replacement therapy and use of local or topical vasoactive medications also excluded subjects from participation. One study eye was included in each volunteer. The right eye was selected unless contraindicated by a unilateral ocular condition, in which case the left eye was used instead.

The study was approved by the Committee for Biomedical Research Ethics in the Capital Region of Denmark. Written informed consent was obtained from all participants prior to entry into the study which adhered to the tenets of the Declaration of Helsinki and was prospectively registered at www.clinicaltrials.gov (NCT01721811).

Methods

Participants underwent measurement of best-corrected visual acuity by the Early Treatment of Diabetic Retinopathy Study (ETDRS) charts, slit lamp biomicroscopy, Goldmann applanation tonometry, dilated ophthalmoscopy, fundus photography (TopCon TRC50 DX; TopCon Corporation, Tokyo, Japan), spectral-domain optical coherence tomography (OCT; Spectralis, Heidelberg Engineering, Heidelberg, Germany and Cirrus SD-OCT; Carl Zeiss Meditec, Jena, Germany) and measurement of axial length by laser interferometry (IOL Master; Carl Zeiss Meditec). Participants underwent a physical examination with special focus on the pulmonary and cardiovascular systems, measurement of brachial blood pressure (Microlife BP A80; Microlife AG, Widnau, Switzerland), height and weight. Mean arterial pressure (MAP) was calculated as

$$\mathrm{MAP} = \frac{2}{3} \times \text{diastolic blood pressure} + \frac{1}{3} \times \text{systolic blood pressure}$$

and body mass index (BMI) as

$$\mathrm{BMI} = \frac{\text{weight (kg)}}{\text{height (m)}^2}$$

Study procedures

The oxygen reactivity test was performed at the second of two study visits. Study procedures were conducted in the morning after an overnight fast, which also included abstinence from caffeine-containing beverages, and following pupil dilation to a diameter of at least 7 mm using topical 1% tropicamide and 10% phenylephrine hydrochloride (except for intraocular pressure). Procedures included venous blood samples, OCT, applanation tonometry, capillary blood glucose, weight, blood pressure, pulse, dual-wavelength retinal oximetry and motion-contrast fundus photography.

Blood samples

Venous blood samples were obtained in the morning after an overnight fast. Analyses included blood haemoglobin and haemoglobin A1c (HbA1c), plasma (p-) creatinine, p-sodium, p-potassium, p-total, high-density lipoprotein and low-density lipoprotein cholesterol and p-triglycerides.

During the oxygen reactivity test, arterial blood samples were obtained from the radial artery at two time-points, one before ($n = 15$) and one during hyperoxia ($n = 13$). Analyses included arterial blood haemoglobin oxygen saturation, partial pressures of O_2 (pO_2) and CO_2 (pCO_2), pH, base excess and the concentrations of sodium, potassium, ionized calcium and glucose. All blood samples were analysed at the laboratory of the Glostrup Hospital using the ABL800 Flex, Radiometer Medical ApS, Bronshoj, Denmark, the Vitros 5,1FS/5600, Ortho-Clinical Diagnostics Inc., Rochester, NY, USA and the Advia 2120i, Siemens Healthcare AG, Erlangen, Germany.

Capillary blood glucose (FreeStyle Precision; Abbott Diabetes Care, Witney, UK) was measured at baseline at each study visit ($n = 16$ and $n = 13$ at visit 1 and 2, respectively).

Retinal oximetry and retinal trunk vessel diameters

Dual-wavelength retinal oximetry was performed using the T1 Retinal Oximeter (Oxymap ehf., Reykjavik, Iceland). Three 50° oximetry images centred on the optic disc were captured at each time-point. Using proprietary software (OXYMAP ANALYZER V. 2.4.0), the widest arterial and venous segment supplying each retinal quadrant were identified at a distance of 0.5–1.0 disc diameter from the optic disc margin by a single observer. The software estimated the average diameter (Blondal et al. 2011) and measured the optical density ratio (ODR) of individual vessel segments (Geirsdottir et al. 2012). Using the Oxymap Analyzer and parameters supplied by the manufacturer, oxygen saturation estimates were calculated by linear conversion from ODR and were automatically adjusted for vessel diameter using linear correction to avoid the artefactual effect of vessel diameter on saturation measurements (Geirsdottir et al. 2012). Vessel diameters and oxygen saturations were averaged across the three images at each time-point, yielding summary measures for retinal arteries and veins, respectively. Vessel segments were at least 6 pixels wide and 50 pixels long. An artery or a vein was only included if a corresponding vessel of the opposite type was also identified in the same quadrant. This enabled calculation of the arteriovenous oxygen saturation difference (AVD) as an estimate of retinal oxygen extraction:

$$\text{AVD} = \text{Mean arterial saturation} - \text{Mean venous saturation}$$

Macular perfusion velocities

Macular perfusion velocities were measured by the Retinal Function Imager 3005 (RFI; Optical Imaging Inc., Rehovot, Israel) (Izhaky et al. 2009; Landa et al. 2009; Burgansky-Eliash et al. 2010, 2014; Beutelspacher et al. 2011). Motion-contrast fundus photography included at least three sets of eight sequential red-free 20° macula-centred fundus images acquired at interframe intervals of 17.5 ms. Image capture was pulse synchronized by a pulse oximetry probe attached to the participant's finger, allowing all image series to be acquired during the cardiac diastole. Images were processed by a single observer using proprietary software (RFI BROWSE, version 2.0.2.159; Optical Imaging Inc.). After exclusion of offset images, image series were included in the flow velocity analysis if they were well centred on the fovea and contained at least four consecutive images with sufficient macular flow information. An ETDRS macular grid (Early Treatment Diabetic Retinopathy Study Research Group 1991) was centred on the fovea using manual overlay, and vessel segments approximately 100–150 pixels in length were identified between the innermost and second ring of the ETDRS grid, that is, between 500 and 1500 μm from the centre of the fovea. Vessel segments were marked using an operator-supervised path constraint method (Nelson et al. 2005). Vessel crossings were avoided while vessel segments passing a major branching were divided at this point. The vessel pattern template thus produced was cross-correlated across all eligible image series (minimum of three series) from the same eye at a given recording session. The median linear flow velocity was calculated for each vessel segment, and these values were averaged for arteries and veins, respectively. For subsequent imaging sessions, the baseline vessel template was used again with automated image alignment by the RFI software.

For each imaging session of the oxygen reactivity test, the software used blood flow information to create perfusion maps delineating the paths of blood flow (Izhaky et al. 2009; Witkin et al. 2012). The widths of these paths were taken to represent blood column diameters. Vessel segments corresponding to those used for blood velocity analysis were marked manually. Vessel contours were identified and the diameter of each vessel segment calculated automatically (Taarnhoj et al. 2006). The average diameters of perifoveal arterioles and venules were calculated. Assuming a circular cross-sectional area of the blood vessels, which can be determined as

$$A = \pi \times r^2 = \pi \times \frac{D^2}{4}$$

the average perfusion velocity (v) in mm/second, a conversion factor of 60 second/min and the average vessel diameter (D) in mm were used to calculate an index of volumetric blood flow (Q) in microliters/min in all participants for each imaging session of the oxygen reactivity test:

$$Q = v \times \pi \times \frac{D^2}{4} \times 60$$

Hyperoxia

Retinal vessel diameters, oxygen saturations and macular flow velocities were measured before, during and after hyperoxia in 16 participants (Fig. 1). Initially, two measurements with participants breathing ambient room air were performed 15 min apart. The second measurement was followed by an arterial blood sample. After a period of rest (15–43 min), participants were connected to a high-flow oxygen delivery system (F&P Optiflow Nasal Cannula and F&P 850 System; Fisher & Paykel Healthcare, Auckland, New Zealand). With participants still breathing ambient room air, a prehyperoxic measurement was performed and this was immediately followed by hyperoxia. A constant flow of 30 l O_2/

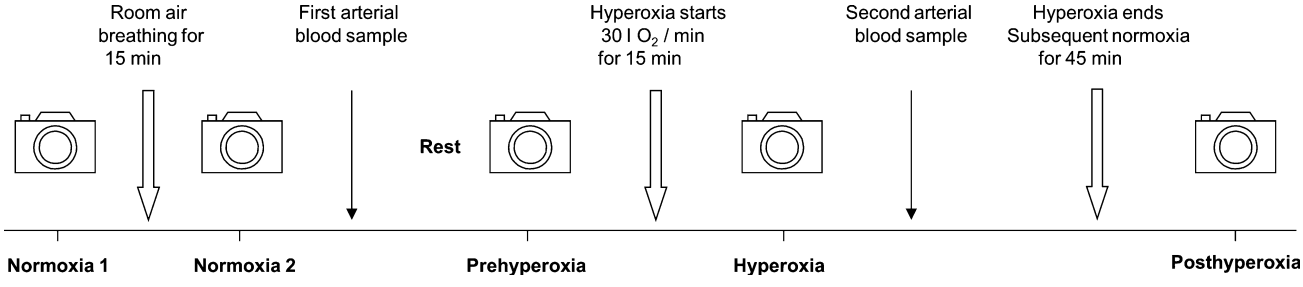

Fig. 1. Flow chart of the oxygen reactivity test. The five experimental conditions are denoted in bold letters with camera symbols representing fundus image captures. White arrows denote time intervals during which participants were exposed to normoxia or hyperoxia. Black arrows represent time-points for the collection of arterial blood samples.

min was passed through a combined heater and humidifier before being delivered to the participant through a double-bore nasal cannula. After 15 min of hyperoxia, the participant underwent fundus photography again followed by a second arterial blood sample. After breathing ambient room air for another 45 min, the final set of fundus images was acquired. Pulse and blood pressure were measured following each set of fundus photographs. Systemic arterial oxygen saturation was measured by fingertip pulse oximetry (Pulse Oximeter; Beijing Choice Electronic Tech. Co. Ltd., Beijing, China). One participant did not complete the posthyperoxic measurement.

Baseline associations and repeatability

Analyses of correlations between outcome variables and associations with anthropometric and biochemical variables were based on the first normoxic measurement of the oxygen reactivity test. Short-term repeatability was evaluated in 16 participants using the first two normoxic measurements of the oxygen reactivity test. Long-term repeatability was investigated in 16 participants who attended two study visits 1–102 days apart (median 44 days). Due to the apparent development of epiretinal fibrosis in the study eye between the two visits, one participant had missing perfusion velocity values at visit 1. The unaffected fellow eye was used to assess oxygen reactivity, and retinal oximetry was available from both eyes at visit 1. One additional participant had missing perfusion velocity values at visit 1 due to technical issues.

Statistical analysis

Statistical analyses were performed by SAS 9.1; SAS Institute, Cary, NC, USA. Results are shown as mean ± SD unless otherwise indicated. The results of the oxygen reactivity test were evaluated by mixed model analysis for repeated measures with Tukey-adjusted comparisons of individual experimental conditions. Repeatability was evaluated by the intraclass correlation coefficient (ICC) and limits of agreement. Paired *t*-test or, in the case of non-normally distributed variables, the nonparametric paired Wilcoxon test was used to evaluate systematic differences between repeated measurements. The ICC and limits of agreement were derived by variance component analysis. ICC was interpreted as previously described (Fleiss 1999). The association between continuous variables was tested by linear regression. The level of statistical significance was set at $p < 0.05$.

Results

Baseline observations

At the normoxic baseline (Table 2), arterial and venous perfusion velocities ($r = 0.90$, $p < 0.0001$; linear regression) and oxygen saturations ($r = 0.76$, $p = 0.0006$) were highly correlated. Arterial and venous perfusion velocities were associated with the respective oxygen saturations ($r = 0.61$, $p = 0.012$; $r = 0.78$, $p = 0.0004$). Major arterial and venous diameters were not associated mutually or with macular perfusion velocities. Higher haemoglobin concentrations tended to be associated with lower arterial ($r = -0.53$, $p = 0.044$) and venous ($r = -0.48$, $p = 0.072$) perfusion velocities, lower venous saturation ($r = -0.71$, $p = 0.0028$) and higher arteriovenous saturation difference ($r = 0.66$, $p = 0.0070$). Outcome variables were not associated with perifoveal vessel diameters, age, BMI, axial length, intraocular pressure, blood pressure, pulse, blood glucose, haemoglobin A1c or p-cholesterol.

Oxygen reactivity

Outcome variables did not change significantly during normoxia (Fig. 2). Fifteen minutes of hyperoxia was accompanied by a mean reduction in arterial velocities of 14%, from 4.2 ± 1.0 to 3.6 ± 0.8 mm/second ($p = 0.0080$; Tukey-adjusted comparison), and in venous velocities of 16%, from 3.1 ± 0.8 to 2.6 ± 0.4 mm/second ($p = 0.0019$). Major retinal arteries constricted by 5.5%, from 114.2 ± 10.7 μm to 107.9 ± 11.3 μm ($p < 0.0001$), and veins constricted by 8.2%, from 147.3 ± 17.1 μm to 135.3 ± 17.2 μm ($p < 0.0001$). Retinal arterial oxygen saturation increased from 95.1 ± 5.0% to 96.6 ± 6.4% ($p = 0.038$) and venous oxygen saturation from 62.9 ± 6.7% to 70.3 ± 7.8% ($p = 0.0010$). All parameters had returned to baseline values 45 min after reverting to room air breathing. Perifoveal vessel diameters did not change significantly during the experiment ($p = 0.13$; $p = 0.49$; overall effect in repeated measures analysis). Changes in the index of volumetric blood flow closely resembled those of the linear macular perfusion velocities with average reductions during hyperoxia of 14.8% ($p = 0.0019$) and 16.6% ($p = 0.0006$) in arterial and venous vessels, respectively.

Hyperoxia increased systemic arterial pO_2 4.1-fold from 13.8 ± 1.6 kPa to 56.6 ± 10.6 kPa ($p < 0.0001$; paired *t*-test) and raised arterial haemoglobin oxygen saturation from 97.6 ± 0.8 % to 100.0 ± 0.0 % ($p < 0.0001$). Concomitantly, pCO_2 was reduced from 5.0 ± 0.6 kPa to 4.4 ± 0.9 kPa (−12%, $p = 0.0007$), arterial blood

Table 2. Absolute values and repeatability of retinal vascular measurements.

Variable	Visit 1	Visit 2 Normoxia 1	Visit 2 Normoxia 2	ICC short term	LoA short term	ICC long term	LoA long term
Arterial perfusion velocity (mm/second)	4.0 ± 0.9	4.2 ± 0.9	4.1 ± 0.9	0.82	±1.1 (mm/second)	0.11	±2.4 (mm/second)
Venous perfusion velocity (mm/second)	3.2 ± 0.7	3.2 ± 0.7	3.1 ± 0.7	0.79	±0.9 (mm/second)	0.06	±1.8 (mm/second)
Central arterial diameters (μm)	114.1 ± 11.3	114.1 ± 12.3	113.8 ± 10.7	0.93	±8.6 (μm)	0.91	±10.0 (μm)
Central venous diameters (μm)	149.8 ± 18.0	148.5 ± 17.5	147.0 ± 17.8	0.98	±6.8 (μm)	0.97	±9.2 (μm)
Arterial saturation (%)	94.6 ± 5.5	94.8 ± 5.6	95.1 ± 5.0	0.98	±1.9 (%)	0.97	±2.9 (%)
Venous saturation (%)	62.7 ± 6.3	62.9 ± 6.6	62.5 ± 6.9	0.98	±2.9 (%)	0.93	±4.7 (%)
Arteriovenous saturation difference (%)	31.9 ± 3.5	31.9 ± 4.3	32.6 ± 4.3	0.92	±3.6 (%)	0.88	±3.8 (%)

Mean values of retinal vascular measures ±SD in 16 healthy volunteers at 2 study visits performed 1–102 days apart [Visit 1 and the first normoxic measurement at visit 2 (Normoxia 1)]. These were used to assess long-term repeatability. The first two measurements of the oxygen reactivity test (Normoxia 1 and Normoxia 2) were performed 15 min apart at visit 2 and were used to assess short-term repeatability. Intraclass correlation coefficients (ICCs) and limits of agreement (LoA) were derived from variance component analysis. LoA have the same units as the mean values, whereas ICCs are dimensionless coefficients.

alkalinized from pH 7.41 ± 0.03 to 7.45 ± 0.06 ($p = 0.0009$) and ionized calcium decreased from 1.19 ± 0.02 mmol/l to 1.18 ± 0.03 mmol/l ($p = 0.020$). Base excess, bicarbonate, lactate, glucose, haemoglobin, potassium and sodium did not change in relation to hyperoxia. Arterial oxygen saturation as measured by fingertip pulse oximetry increased from 97.7 ± 1.1 % at baseline to 99.5 ± 0.6 % during hyperoxia ($p = 0.0015$) and returned to 98.1 ± 1.3 % during post-hyperoxic normoxia ($p = 0.67$ versus baseline; $p = 0.011$ versus hyperoxia). In participants who did not have arterial blood samples drawn during hyperoxia, pulse oximetry revealed increases of systemic arterial oxygen saturation to 100% in two participants and to 99% in one participant. Pulse and blood pressure did not change significantly during the experiment.

The effects of hyperoxia on arteriolar and venular perfusion velocities were positively associated mutually ($r = 0.79$, $p = 0.0003$) and negatively associated with the respective prehyperoxic perfusion velocities (arterial $r = -0.56$, $p = 0.024$; venous $r = -0.88$, $p < 0.0001$; Fig. 3). The latter associations tended to remain significant when relative changes in perfusion velocities were analysed (arterial $r = -0.44$, $p = 0.089$; venous $r = -0.81$, $p = 0.0001$). Only the hyperoxic change in retinal arterial saturation was positively associated with the prehyperoxic measurement ($r = 0.65$, $p = 0.0061$). Changes in perfusion velocities were associated with changes in the diameters of major retinal veins ($r = 0.56$, $p = 0.024$; $r = 0.57$, $p = 0.021$).

Repeatability

Retinal trunk vessel diameters, oxygen saturations and the arteriovenous saturation difference had excellent short-term and long-term repeatability (Table 2). The primary analysis showed that macular perfusion velocities had good short-term but poor long-term repeatability. A secondary analysis revealed that changes in perfusion velocities between study visits were negatively associated with spontaneous changes in blood glucose ($r = -0.73$; $p = 0.0067$ and $r = -0.67$; $p = 0.018$; Fig. 4), which accounted for 53% and 45%, respectively, of the variation in perfusion velocities. Changes in blood haemoglobin tended to be negatively associated with changes in venous saturation ($r = -0.49$, $p = 0.062$). Changes in arterial and venous perfusion velocities were associated both within ($r = 0.75$, $p = 0.0008$) and between examination sessions ($r = 0.88$, $p < 0.0001$). Long-term changes in major arterial and venous diameters ($r = 0.58$, $p = 0.019$) and retinal oxygen saturations ($r = 0.59$, $p = 0.016$) were also associated. No other associations were found between changes in outcome variables between study visits and changes in blood glucose, blood pressure, pulse or systemic arterial saturation.

Discussion

This study of retinal vasodynamics in healthy human volunteers found that 15 min of hyperoxia had significantly reduced macular perfusion velocities, constricted retinal trunk vessels and increased blood oxygen saturations. The study provided proof-of-principle validation of motion-contrast retinal blood velocimetry and supported the validity of an automated retinal vessel diameter measurement (Blondal et al. 2011) and diameter-adjusted dual-wavelength retinal oximetry (Beach et al. 1999; Geirsdottir et al. 2012; Traustason et al. 2013).

The perfusion velocity measurements of the RFI have previously been validated in a laboratory setting using a model eye and fixed flow rates of human blood through a micropipette with an inner diameter of 80 μm (U.S.Food & Drug Administration 2008). In the retinal microcirculation, hyperoxia has resulted in reductions of 13–28% in leucocyte velocity (Sponsel et al. 1992; Kiss et al. 2002; Tomic et al. 2005) and 22% in erythrocyte velocity (Strenn et al. 1997). Blue field entoptic and laser Doppler velocimetry are not commercially available, and it was thus not possible to perform a direct method comparison. Still, the effect of hyperoxia on macular perfusion was qualitatively and

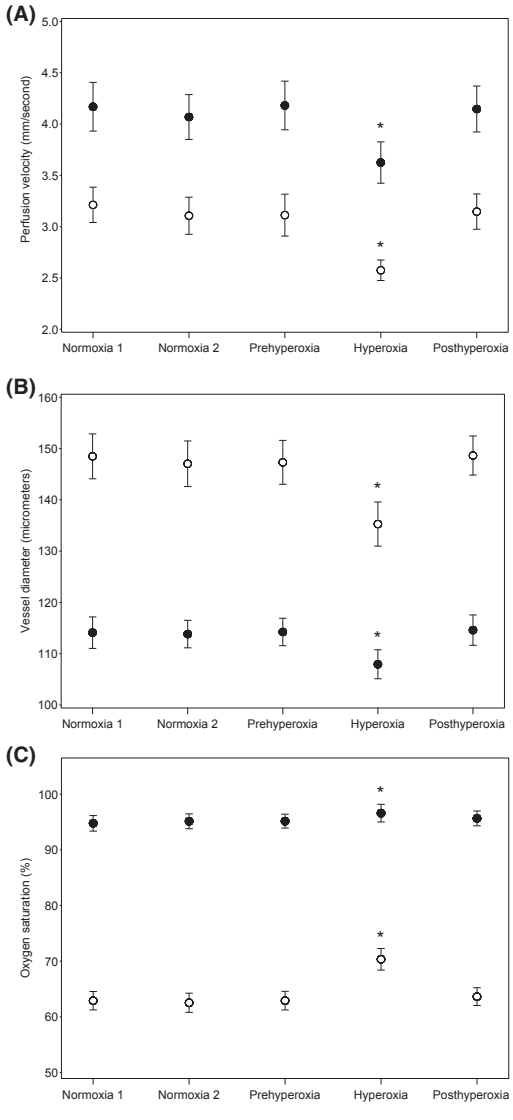

Fig. 2. Retinal hemodynamic changes during the oxygen reactivity test. Macular perfusion velocities (A), retinal vessel diameters (B), retinal haemoglobin oxygen saturations (C) at the five experimental conditions of the oxygen reactivity test (n = 16). Normoxia 1 and Normoxia 2 were separated by 15 min of room air breathing. The prehyperoxic and hyperoxic measurements were separated by 15 min of pure oxygen breathing. The posthyperoxic measurement was performed after 45 min of subsequent normoxia. (●) Arterioles, (○) Venules. Data are presented as means. Error bars represent the standard error of the mean. *p < 0.05 versus prehyperoxic measurement.

quantitatively almost identical in the present study. These results suggest that motion-contrast velocimetry may be considered a useful alternative method for quantifying macular blood flow.

A floor effect was indicated by the observation that hyperoxia could not be expected to reduce the perfusion velocity if the prehyperoxic value was below a certain threshold. The baseline association of high haemoglobin concentrations with low perfusion velocities and vice versa is consistent with findings in the retina (Grunwald et al. 1994) and other microcirculatory systems (Vicaut et al. 1987; Mirhashemi et al. 1988; Colantuoni & Bertuglia 1997) including the central nervous system (Morimoto et al. 2001; Ibaraki et al. 2010).

The good short-term and poor long-term repeatability of macular perfusion velocity measurements as observed in the primary repeatability analysis confirmed previous observations (Yap & Brown 1994; Strenn et al. 1997; Kagemann et al. 1998; Rose & Hudson 2007). This study, however, offered an explanation for the large intra-individual variation as approximately half of the day-to-day variation in perfusion velocities could be ascribed to changes in blood glucose. These results suggest that retinal perfusion is balanced with substrate availability around individual set points. Associations between non-acute changes in blood glucose and retinal blood flow have previously been observed in patients with diabetes (Feke et al. 1994; Grunwald et al. 1994, 1995). Our finding of an inverse relationship between changes in macular perfusion velocities and changes in blood glucose in healthy volunteers is consistent with the observation that initiation of strict metabolic control in patients with diabetes resulted in increased leucocyte velocity in the macular microcirculation (Grunwald et al. 1995). The relationship between blood glucose and retinal blood flow may be more complex, however. Hence, blood flow changes in the major retinal vessels appear to differ from those in the macular microcirculation (Grunwald et al. 1995). Specifically, blood flow in major retinal veins was reduced after initiation of strict metabolic control in patients whose diabetic retinopathy did not progress, whereas a non-significant blood flow increase was observed in patients with later retinopathy progression (Grunwald et al. 1994). Long-term blood flow variability in major retinal arteries in patients with diabetes could mainly be ascribed to changes in centreline blood speed which were positively associated with spontaneous changes in blood glucose (Feke et al. 1994). During acute hyperglycaemia in humans, retinal blood flow has been reported to increase or to remain unchanged (Sullivan et al. 1991; Bursell et al. 1996; Kida et al. 2002; Gilmore et al. 2007b). Other potential sources of variability include the camera-to-eye distance (Kagemann et al. 1998), image focus, eye movements and limitations of the image alignment algorithms of the RFI.

The RFI field-of-view was 20° instead of the previously used 35° (Burgansky-Eliash et al. 2013) which improved the resolution of the microcirculation but with smaller depth of field and larger sensitivity to eye movements. Image centring and vessel segment selection were standardized. Previous studies excluded vessel segments with intrasession coefficients of variation >0.45 (Landa et al. 2012; Burgansky-Eliash et al. 2013). Although effective, this approach can

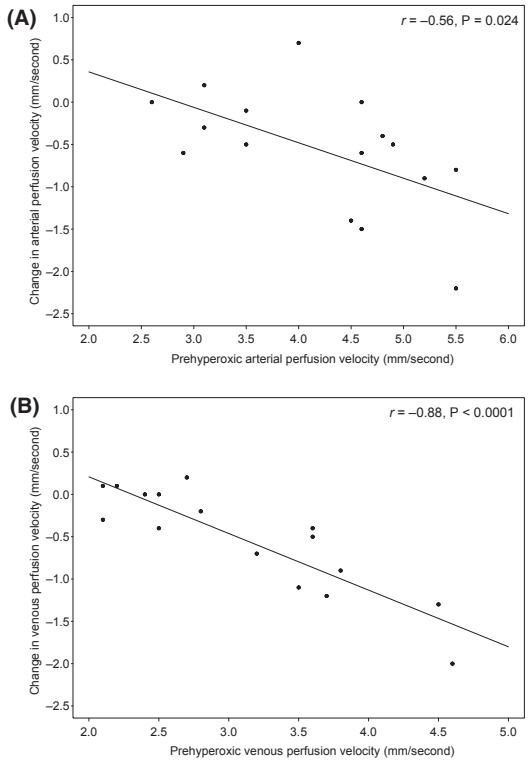

Fig. 3. Hyperoxic changes in macular perfusion in relation to prehyperoxic values. Changes in arterial (A) and venous (B) macular perfusion velocities after 15 min of hyperoxia in relation to prehyperoxic perfusion velocities (n = 16).

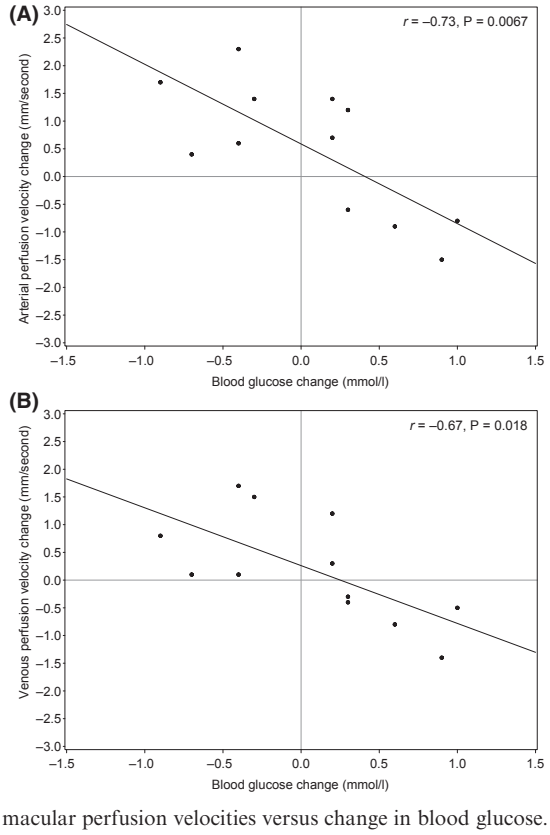

Fig. 4. Change in macular perfusion velocities versus change in blood glucose. Changes in retinal arterial perfusion velocities (A) and retinal venous perfusion velocities (B) in 12 healthy volunteers in relation to changes in capillary blood glucose between two separate examination sessions 1–102 days apart.

be sensitive to outliers, and it can be difficult to ensure that the same vessel segments are analysed in repeated imaging sessions. To enable intra-individual comparisons over time, averaging of the median values of all vessel segments is suggested as an alternative statistic.

The RFI measures linear perfusion velocities and not volumetric flow. Perifoveal vessel diameters, as measured in perfusion maps, were not affected by hyperoxia. Therefore, it was not surprising that the oxygen reactivity of the index of volumetric flow closely resembled that of macular perfusion velocities. The absolute values of arterial and venous flow indices could not be compared because they were not necessarily derived from directly corresponding vessel segments. Although image quality in perfusion maps was often better than in raw images, delineation of vessel borders was not always optimal and diameters ranged from 3 to 9 pixels which reduced accuracy and precision. These observations suggest that perifoveal vessel diameters provided limited additional information and that, using the RFI method, perfusion velocities *per se* could be the most relevant measure of macular blood flow. Because resistance to blood flow is proportional to the fourth power of the vessel radius, it is possible that vessel diameter changes too small to be detected by the method used in this study may have influenced macular perfusion. It is also possible, however, that vessel diameters did not change and that the RFI measured perfusion velocities in conduit vessels rather than resistance vessels. Flow regulation may take place at several locations in the retinal circulation. Vessel constriction can occur along the entire length of a retinal artery (Jeppesen et al. 2007), and pericytes which surround retinal capillaries can contribute to local control of blood flow (Puro 2012).

Velocity measurements are based on tracking of what is presumed to be moving clusters of erythrocytes in consecutive fast-sequence images, the contrast being enhanced by the red-free flashes (Izhaky et al. 2009). Leucocytes influence blood flow velocities in capillaries and small postcapillary venules, but the effect should be negligible in larger vessels (Baskurt & Meiselman 2003; Sugihara-Seki & Fu 2005).

Potentially, the fast-sequence flashes of the RFI could increase retinal blood flow (Riva et al. 2005). However, the high flash frequency, the short duration of flash trains, and the relatively long intervals between flashes (Riva et al. 2005) have probably reduced the effect of flicker on successive measurements.

In the retina, hyperoxia has been shown to constrict major arterioles and venules by 5.6–14.0% and 5.8–15.0%, respectively (Hickam & Frayser 1966; Pakola & Grunwald 1993; Gilmore et al. 2005; Rose & Hudson 2007; Lott et al. 2012). The response in the present study was of comparable magnitude. The study confirmed that repeatability of trunk vessel diameter measurements was excellent (Blondal et al. 2011). Residual intra-individual variation may be related to the pulse cycle (Chen et al. 1994) and spontaneous diameter oscillations (Bek et al. 2013).

Oxygen extraction has previously been found to decrease with increasing haemoglobin concentration (Ibaraki et al. 2010). In this study, the opposite association was found in a cross-sectional analysis of baseline data. It was explained by lower estimated retinal venous saturation, corresponding to higher venous ODR, in participants with a high haemoglobin concentration and vice versa. Hence, haemoglobin could have the same effect on ODR as vessel diameter. Retinal arterial saturation, however, was not related to haemoglobin concentration. It is noteworthy that a previous study found a closer association of saturation estimates and vessel diameter in retinal veins than in retinal arteries (Hammer et al. 2008). This suggests that the artefacts arising from vessel diameter and haemoglobin concentration could have a similar origin. Oxygen extraction depends on retinal blood flow in addition to the arteriovenous saturation difference which was derived from a subgroup of retinal vessels.

It has been suggested that higher blood flow could lead to higher retinal oxygen saturation (Hardarson & Stefansson 2012; Bek 2013). This hypothesis was supported by the association of macular perfusion velocities and retinal oxygen saturations at the normoxic baseline in the present study. Optimally, this finding, which is consistent with observations during experimental changes of ocular perfusion (Beach et al. 2007), should be tested by concomitant measurement of oxygen saturation and blood flow in the same retinal vessels. Interindividual variation, normoxic calibration, reduced vessel diameters and possibly vessel wall thickness (Harris et al. 2003) may explain why mean retinal arterial saturation did not reach 99–100% during hyperoxia in the present study. The contribution of the two latter variables could be limited, however. Because of the relatively large interindividual variation of retinal oximetry measurements (Hardarson et al. 2006; Hammer et al. 2008), part of which may be related to differences in fundus pigmentation (Beach et al. 1999; Hammer et al. 2008) and limitations of the calibration (Harris et al. 2003), it has been cautioned that results of dual-wavelength retinal oximetry should not be regarded as absolute values (Harris et al. 2003). The change in mean retinal arterial oxygen saturation during hyperoxia in the present study was slightly smaller than that observed by Hammer et al. (Hammer et al. 2008), and it was consistent with the change in mean systemic arterial saturation. Larger increases in retinal venous than arterial saturation could be caused by the ability of oxygen diffusion from the choroid to supply a larger proportion of the retina with consequent reductions in oxygen extraction from the inner retinal circulation (Dollery et al. 1969) and smaller oxygen saturation reductions in the capillaries because of increased concentrations of physically dissolved oxygen.

High-flow oxygen delivery via nasal cannula ensured a rapid, substantial increase in arterial pO_2 and a high degree of participant comfort (Dysart et al. 2009; Ward 2013). This method is used clinically to treat patients with hypoxemic respiratory failure and can provide higher inspiratory fractions of O_2 than a non-rebreathing oxygen mask at comparable flow rates (Ward 2013). A potential limitation is that the hyperoxia was not isocapnic. The high oxygen flow rates lead to washout of the anatomical dead-space and increased alveolar ventilation (Ward 2013) which, in turn, reduced arterial pCO_2 and increased pH. Relative hypocapnia, however, would be expected to change retinal vessel diameters and blood flow velocities in the same direction as relative hyperoxia (Hickam & Frayser 1966; Tsacopoulos & David 1973; Venkataraman et al. 2008). Systemic pO_2 and retinal vascular measures stabilize after 10–15 min of hyperoxia (Struder et al. 1999; Gilmore et al. 2005; Jean-Louis et al. 2005). Hence, the only hyperoxic measurement in this study was presumably performed under steady-state conditions.

Although hyperoxia leads to reproducible retinal vascular responses and is therefore useful for validation of new measurement methods, it does not reflect a physiological situation, and studies of retinal hypoxia may be more relevant to understand the pathophysiology of retinal vascular disease. Still, retinal vascular reactivity to hyperoxia can detect abnormal autoregulation in patients with diabetes (Grunwald et al. 1984; Fallon et al. 1987; Gilmore et al. 2007a; Justesen et al. 2010; Lott et al. 2012) Although the present study only included healthy participants, the results highlighted a need to include baseline macular perfusion velocities in the interpretation of hyperoxic reactivity testing. Recent studies have shown that retinal vascular responses to flicker stimulation can be used as an alternative (Mandecka et al. 2007; Lott et al. 2012) and that retinal oximetry findings differ between disease stages in diabetic retinopathy and maculopathy (Jorgensen et al. 2014). The predictive values, however, remain to be determined in longitudinal studies. In the present study of healthy individuals, oximetry results were highly repeatable and the variation was not associated with fluctuations in blood glucose. In contrast, acute hyperglycaemia in patients with diabetes reduced retinal venous oxygen saturation (Tiedeman et al. 1998).

The required pupil dilation for retinal imaging was achieved by topical tropicamide and phenylephrine hydrochloride. Tropicamide does not affect macular capillary flow (Robinson et al. 1985), whereas blood flow may decrease in parapapillary vessels (Harazny et al. 2013). Instillation of phenylephrine in addition to tropicamide does not influence retinal vessel diameter and oximetry measurements (Vandewalle et al. 2013) or changes in retinal vessel diameters, blood flow, and blood velocities during hyperoxia (Tsui et al. 2013).

The statistical methods used to analyse the primary outcomes of the oxygen reactivity test were chosen to minimize the risk of a type 1 error. Because multiple testing increases the risk of detecting significant findings by chance, the results of the univariate linear regression analyses should be interpreted with caution. Still, the validity of the statistically significant findings in this study is supported by the fact that they were in accordance with previous observations and, in most cases, consistent in arteries and veins.

In conclusion, the study supports that motion-contrast velocimetry is a useful alternative to blue field entoptic and laser Doppler velocimetry. Multimodal, non-invasive characterization of retinal vascular function has the potential to provide a comprehensive overview of retinal metabolism. Ancillary findings suggested that retinal perfusion is regulated around individual set points according to the blood glucose levels.

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