Systemic circulatory influences on retinal microvascular function in middle-age individuals with low to moderate cardiovascular risk

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Total word count: 8181

Tables & Figures: 2 tables and 3 figures

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Abstract

Purpose: To investigate the relationship between retinal microvascular reactivity, circulatory markers for CVD risk and systemic anti-oxidative defence capacity in healthy middle-aged individuals with low to moderate risk of CVD.
Methods: Retinal vascular reactivity to flickering light was assessed in 102 healthy participants (46-60 years) by means of dynamic retinal vessel analysis (DVA). Other vascular assessments included carotid intima-media thickness (C-IMT) and blood pressure (BP) measurements. Total cholesterol (CHOL), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), triglycerides (TG) and blood glutathione levels in its reduced (GSH) and oxidized (GSSG) forms were also determined for each participant, along with Framingham risk scores (FRS).
Results: Retinal arterial baseline diameter fluctuation (BDF) was independently, significantly and negatively influenced by LDL-C levels (β=-0.53, p=0.027). Moreover, the arterial dilation slope ($Slope_{AD}$) was independently, significantly and positively associated with redox index (GSH: GSSG ratio, β=0.28, p=0.016), while the arterial constriction slope ($Slope_{AC}$) was significantly and negatively influenced by blood GSH levels (β=-0.20, p=0.042), and positively associated with FRS (β=0.25, p=0.009). Venous BDF and dilation amplitude (DA) were also negatively influenced by plasma LDL-C levels (β=-0.83, p=0.013; and β=-0.22, p=0.028, respectively).
Conclusions: In otherwise healthy individuals with low to moderate cardiovascular risk, retinal microvascular dilation and constriction responses to stress levels are influenced by systemic antioxidant capacity, and circulating markers for cardiovascular risk.

Key words: oxidative stress, retina, vascular function, cardiovascular risk, dynamic retinal vessel analysis

Introduction

Functional assessments of microvessels are of particular clinical interest since endothelial dysfunction, one of the main culprits for the development of atherosclerosis, is thought to occur much earlier at the microvascular than at the macrovascular level (Gariano & Gardner 2005; Gates et al. 2009). Consequently, several methods have been developed to assess functional responses in various microvascular beds. Among those, dynamic retinal vessel analysis (DVA) was recently identified as a useful measure of early changes that signal endothelial dysfunction and risk for future cardiovascular pathologies in individuals with and without overt disease (Nagel & Vilser 2004; Nagel et al. 2006; Mandecka et al. 2007; Bek et al. 2008; Heitmar et al. 2010; Patel et al. 2011; Mroczkowska et al. 2012; Patel et al. 2012; Qin et al. 2013). This is generally possible due to the fact that the retinal microvascular response to flicker provocation is, in part, dependent on nitric oxide (NO) release, (Dorner et al. 2003) and compromised NO homeostasis is known to be a key factor in endothelial dysfunction at all vascular levels. However, other mechanisms such as altered metabolic demand and neurovascular coupling are also largely involved in the retinal vascular response to flickering light (Lim et al. 2012). Indeed, we have already demonstrated that retinal microvascular function as measured by DVA is affected in patients with Alzheimer's disease, a pathological process largely associated with disturbed neurovascular coupling. (Mroczkowska et al. 2013). Nevertheless, it has been hypothesized that abnormal neurovascular coupling in such context could also represent a direct consequence of endothelial dysfunction (Lim et al. 2012).

At the systemic level it is known that degradation of NO by free radicals generated during oxidative stress may impair vasodilation (Cines et al. 1998), and therefore result in vascular dysfunction. The human body uses a complex anti-oxidative defence mechanism involving glutathione among other factors to combat stressors such as high oxygen flux. Consequently, any condition associated with low levels of circulating glutathione result in a higher rate of oxidative reactions that contribute towards low NO bioavailability, with important consequences on the normal regulation of systemic haemodynamics (Vallance & Chan 2001). In parallel, it is also known that abnormal lipid metabolism and insulin resistance are linked to microvascular endothelial dysfunction and increased cardiovascular disease (CVD) risk. The combined effect of low glutathione and abnormal circulating lipids could therefore amplify endothelial dysfunction at all vascular levels, including the retinal vessels (Patel et al. 2011; Qin et al. 2013). This possibility may be important for screening and early intervention, particularly in individuals free of overt vascular disease, but who belong to a group when age-induced cardiovascular complications start to occur. Indeed previous reports indicate that nearly 50% of individuals who suffer a fatal cardiovascular event had not received a positive diagnosis or displayed symptoms prior to death (Gallino 2012). Despite this, traditional risk factor estimates such as Framingham risk scores (FRS) are still largely used for disease prognosis. It is known, however, that the FRS can either over- or under-estimate actual risk in a large number of individuals (Cohn 2013). As other more sensitive biomarkers emerge (Vasan 2006; Helfand et al. 2009; Wang 2011; Ge & Wang 2012), it seems that using these in addition to the FRS could increase the likelihood of early CVD detection (Wannamethee et al. 2005; Mattace-Raso et al. 2006; Fowkes et al. 2008; Kavousi et al. 2012). This represents an important need in the current concept of prediction, prevention, and personalised interventions that target individualized risk for certain diseases (Harvey et al. 2012). Following the trend of modern research, cardiovascular medicine has also embraced the study of "omics" (genomics, metabolomics, proteomics) (Ge & Wang 2012) where detailed biological profiling of individuals rather than population groups has been crucial in initiating the move to personalised medicine. Since biomarkers that form an important component of these strategies comprise anything from genetic markers to imaging tests, functional microvascular assessments could also provide integrated and dynamic data to aid in establishing a new CVD risk indicator for each individual. We have already demonstrated that assessing retinal microvascular function is an

easy, non-invasive method demonstrated to offer early detection of vascular risk in asymptomatic individuals (Patel et al. 2011). Nevertheless, a better understanding of a possible relationship between established biomarkers of CVD risk, and microvascular retinal function in individuals with low to intermediate CVD risk is still needed. The aim of the present study is therefore to investigate the influences that circulatory markers for CVD risk and systemic anti-oxidative defence capacity have on retinal microvascular reactivity, in healthy middle-aged individuals with low to intermediate risk of CVD.

Methods

Study participants

Healthy individuals over the age of 40 were recruited through advertisements at the Vascular Research Laboratory, Aston University (Birmingham, UK) for inclusion in this prospective study. Ethical approval was sought from the relevant local ethics committee and written informed consent was received from all participants prior to study enrollment. The study was designed and conducted in accordance with the tenets of the Declaration of Helsinki and all study-related procedures adhered to institutional guidelines.

Study exclusion criteria were defined as the positive diagnosis of cardiovascular disease, (coronary artery disease, heart failure, arrhythmia, stroke, transient ischaemic attacks), cerebrovascular disease, peripheral vascular disease, severe dyslipidaemia (defined as plasma triglycerides > 6.00mmol/L or cholesterol levels > 7.00mmol/L), diabetes, as well as other metabolic disorders or chronic diseases that required treatment. Frequent use of vasoactive medications such as dietary supplements containing vitamins or antioxidants and bronchodilators also served as exclusion criteria. Potential participants were also screened for ocular diseases and were excluded from the study if they had a refractive error of more than ±3DS and more than ±1DC equivalent, intra-ocular pressure (IOP) greater than 21 mmHg, cataract or any other media opacities, as well as history of intraocular surgery or any form of retinal or neuro-ophthalmic disease affecting the ocular vascular system.

General investigations

Participants who met the inclusion criteria were requested to complete a general health history questionnaire, also detailing daily diet, physical activity, and alcohol consumption. All study-related measurements were performed between 8 and 11 am following a 12-hour overnight fast, which included refraining from alcohol and caffeine.

Standard anthropometric measures of height and weight were recorded to determine body mass index (BMI= weight/height^2). Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were measured using an automatic BP monitor (UA-767, A & D Instruments Ltd, UK) and IOP readings were obtained using non-contact tonometry (Keeler Pulsa*ir*, UK).

Carotid intima-media thickness

Intima-media thickness measurements of the left and right common carotid arteries were obtained for all participants in accordance with an already published protocol (Salonen et al. 1991). Briefly, each patient was positioned in supine position and a high-resolution B-mode ultrasound system (Siemens, Acuson-Sequoia, UK) was used to capture an image of the right then left carotid artery at the level of the carotid bifurcation. C-IMT measurements were then taken from the central region of the inferior arterial wall using the in-built software caliper system, at a site proximal to the artery bifurcation.

Dynamic retinal vessel analysis

Retinal vessel reactivity was assessed using the dynamic retinal vessel analyser (DVA, IMEDOS GmbH, Jena, Germany) in accordance with an established protocol (Nagel et al. 2005). All measurements were performed in a temperature controlled environment (22^{o}C) following pupil dilation with 1% Tropicamide (Chauvin Pharmaceuticals Ltd, UK), and were taken from the inferior temporal vessel branches approximately one and a half disc diameters from the optic nerve head of one unselected eye. The flicker stimulation protocol involves a 350-second continuous diameter recording along short 1mm sections of the retinal vasculature, the duration of which included a 50-second baseline measurement followed by three successive cycles of flicker stimulation distinguished as a 20-second stimulus (opto-electronically generated at 12.5Hz) and an 80-second recovery period (still illumination). The following retinal vessel reactivity and time course parameters, collectively known as Sequential and Diameter Response Analysis (SDRA) (Heitmar et al. 2010), were then calculated. The differences between the maximum and minimum baseline vessel diameter was termed as baseline-diameter fluctuation (BDF), the maximum diameter (MD) was used to describe the maximal vessel dilation in response to flicker light stimulation expressed as a percentage relative to baseline, the time taken (seconds) to reach the maximum vessel diameter during flicker exposure was termed as MD reaction time (tMD), the minimal vessel diameter during the recovery period was used to determine the maximum vessel constriction and calculated as a percentage relative to baseline and expressed as the maximum constriction (MC) whilst the time taken (seconds) to reach maximal vessel constriction was termed maximum constriction reaction time (tMC), and the difference between maximal dilation and constriction responses was termed as the dilation amplitude (DA). In addition, the nature of the entire dynamic vessel response profile for both arteries (A) and veins (V) was explored using high-level programming language functions in Matlab (Version 2010a; MathWorks Inc., Natick, MA), as detailed previously (Mroczkowska et al. 2012), to determine dilation slope ($Slope_{AD/VD}$ = MD-baseline diameter/tMD) and constriction slope ($Slope_{AC/VC}$ = MC-MD/tMC), defined as the interaction between the change in vessel diameter and the rate at which this change occurs.

Biomarker Assays

Bloods samples drawn from the ante-cubital fossa vein on the morning of the appointment were collected into standard EDTA Vacutainer ® tubes and assessed immediately for fasting triglycerides (TG), plasma total cholesterol (CHOL) and high-density lipoprotein cholesterol (HDL-C) using the Reflotron Desktop Analyser (Roche Diagnostics, UK). Low-density lipoprotein cholesterol (LDL-C) values were calculated as per the Friedewald equation (Friedewald et al. 1972).

Glutathione recycling assay

Blood collection, initial processing, and processing for the glutathione recycling assay were as detailed previously (Gherghel et al. 2013). Briefly, a 30µL aliquot of EDTA blood was pre-treated with 33.3 µL of 100 mg/mL 5-sulfosalicylic acid (SSA), 936.7µL sodium phosphate buffer (pH 7.5) to release GSH via cellular disruption and protein precipitation. The sample was centrifuged at 13,000 rpm for 5 minutes, and the supernatant was stored at -80^{o}C for further analyses. Based on previous reports of sample stability, assays were conducted within 2 months of collection (Jones et al. 1998). The GSH levels [t-GSH – (2 x GSSG)] and the redox index (defined as the GSH/GSSG ratio) were determined according to an established enzymatic recycling assay (Tietze 1969; Anderson 1996) that involves the oxidation of GSH by the sulfhydryl reagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB), which can be measured spectrophotometrically at 412 nm. Any GSSG formed was recycled to GSH by glutathione reductase (GSR) in the presence of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH). For the measurement of GSSG levels samples were pre-treated with, 2-vinylpyridine (2VP) in order to derivatize GSH without interfering with GSR reaction. For the

determination of analyte concentrations in the samples, GSH standards were prepared from 0 to 80 µM in increments of 20 µM and GSSG standards were prepared from 0 to 10 µM in increments of 1 µM with the same final concentrations of SSA (1%) as in the samples. Standard curves were generated using a linear regression program (Microsoft Excel, Microsoft Corporation, USA). Since the microplates were read at 0, 1, 2, 3, and 5 minutes, the change in optical density or absorbance at different times points expressed against GSH or GSSG concentration (net reaction rate = slope * GSH or GSSG concentration + intercept) was used to determine GSH or GSSG concentrations in the samples (net rate – intercept / slope * dilution factor). The blood GSH and GSSG concentrations measured in this study were in good agreement with literature data in control patients in the ranges of 150 – 1500 µM and 1 to 500 µM, respectively (Rossi et al. 2002; Gherghel et al. 2005; Gherghel et al. 2013). This suggests that the experimental conditions reported in this study are suitable for the analysis of total glutathione and glutathione disulphide concentrations in whole blood. The validity and reliability of the spectrophotometric method of detection has also previously been established (Rahman et al. 2006).

Framingham Risk Score (FRS)

At present the conventional approach for cardiovascular risk assessment endorses the use of multivariable risk score calculations to estimate an individual's 10-year or lifetime risk of developing CVD. The FRS is a widely used gender-specific algorithm originally developed to estimate CVD risk (Wilson et al. 1987). In the present study FRS was calculated using the current version of the FRS published by an expert panel of the National Heart, Lung and Blood Institute (NHLBI) (2002) and is based on risk factors such as age, gender, CHOL, HDL-C, SBP, treatment for hypertension, smoking status, and diabetes. Risk factors such as age, treatment for hypertension, smoking status and diabetes were identified from self-report questionnaires and CHOL, HDL-C, and SBP values were as those determined on the day of study assessment. The scoring algorithm is based on gender-specific points assigned for each risk factor variable that can be determined using FRS tables i.e. point scores by age group; age group and total CHOL; age group and smoking status; HDL-C level; SBP and treatment status. Ten-year risk percentage is then calculated by total points (1 point, 6%; 2 points, 8%; 3 points, 10%; 4 points, 12%; 5 points, 16%; 6 points, 20%; 7 points, 25%; 10 points or more, > 30%). Absolute CVD risk percentage over 10 years was classified as low risk (< 10%), intermediate risk (10-20%), and high risk (> 20%) (Ford et al. 2004).

Sample Size and Statistical analysis

Based on previous studies, a change of 30% with a SD of 2.5% in retinal vessels reactivity was shown to be significant (Garhofer et al. 2004; Patel et al. 2012). As the study design was multifactorial in nature it was calculated that a sample size of n = 95 was sufficient to provide 95% power at an alpha-level of 0.05.

All statistical analyses were performed using Statistica® software (StatSoft Inc.; Version 9, USA). Distributions of continuous variables were determined by the Shapiro-Wilks test. In cases where normality of the data could not be confirmed appropriate data transformations were made or non-parametric statistical alternatives were used. Group differences were assessed using the Student's t-test for independent variables (normal distributions). Univariate associations were determined using Pearson's (normally distributed) or Spearman's method (non-normally distributed), and forward stepwise regression analyses were performed to test the influence of age, BMI, SBP, DBP, HR, IOP, C-IMT, and circulating markers on the measured variables. Data are expressed for normally distributed variables as mean ± standard deviation (SD) or non-normally distributed variables as median with inter-quartile range (IQR). In multivariate regression models the β coefficient value was considered to answer the question of which of the independent variables has a greater effect on the dependent variable as β refers to the SD change in the dependent

variable per SD increase in the predictor variable, and is particularly useful when variables are measured in different units. A p-value of <0.05 was considered as statistically significant.

Results

One hundred and two healthy participants with low to intermediate global cardiovascular risk (<20 % at 10 years as assessed by the FRS) and similar dietary habits were included in the final analysis.

Table 1 shows the clinical characteristics of the study population, stratified by gender. Men exhibited higher DBP ($p = 0.002$), and FRS ($p < 0.001$), and lower total CHOL ($p = 0.001$), and HDL-C ($p < 0.001$). There were no significant gender differences in age, SBP, HR, IOP, BMI, TG, LDL-C, and C-IMT. Although in our sample, women had lower levels of GSSG ($p = 0.030$), there were no significant gender differences in the redox index (GSH: GSSG), total (tGSH) or reduced glutathione (GSH).

With regards to arterial and venous retinal vascular reactivity parameters as characterized in Table 2, all values reported are based on averaged data across three flicker cycles with the artery and vein regarded separately. There were no significant differences between male and female participants in this study population (all $p > 0.05$).

Univariate analyses revealed that age correlated significantly and negatively with retinal arterial DA ($r = -0.24$, $p = 0.015$), and positively with $Slope_{AC}$ ($r = 0.23$, $p = 0.021$). BMI also correlated negatively and significantly with the retinal arterial BDF ($r = -0.20$, $p = 0.040$). Plasma LDL-C levels correlated negatively and significantly with both arterial BDF and DA ($r = -0.29$, $p = 0.004$; $r = -0.23$, $p = 0.020$, respectively) and venous BDF and DA ($r = -0.24$, $p = 0.018$; $r = -0.22$, $p = 0.028$, respectively). Blood GSH levels correlated significantly and negatively with $Slope_{AC}$ ($r = -0.24$, $p = 0.014$) and positively with overall retinal arterial DA ($r = 0.25$, $p = 0.010$), while the redox index similarly positively influenced $Slope_{AD}$ ($r = 0.32$, $p = 0.002$). The FRS correlated negatively with arterial BDF ($r = -0.21$, $p = 0.035$) and positively with $Slope_{AC}$ ($r = 0.26$, $p = 0.010$) and $Slope_{VC}$ ($r = 0.22$, $p = 0.026$).

Forward stepwise multiple regression analysis revealed arterial BDF to be independently, significantly and negatively influenced by LDL-C ($\beta = -0.53$, $p = 0.027$, Figure 1A) and on the venous side BDF and DA were also independently, significantly and negatively influenced by circulating LDL-C levels ($\beta = -0.83$, $p = 0.013$; $\beta = -0.22$, $p = 0.028$, respectively, Figure 1B and Figure 1C). Also on the arterial side, $Slope_{AD}$ was independently, significantly and positively associated with redox index ($\beta = 0.28$, $p = 0.016$, Figure 2), while $Slope_{AC}$ was significantly and negatively influenced by blood GSH levels ($\beta = -0.20$, $p = 0.042$, Figure 3A), and positively associated with FRS ($\beta = 0.25$, $p = 0.009$, Figure 3B).

Discussion

In the present study we examined the relationship between retinal vessel reactivity, systemic antioxidant defence capacity and circulating markers of cardiovascular risk in individuals free of systemic disease, but with low to moderate FRS for CVD. Systemic 'redox' status (GSH: GSSG) positively influenced retinal arterial dilation capacity ($Slope_{AD}$) during flicker while systemic GSH levels negatively influenced retinal arterial constriction capacity ($Slope_{AC}$). In addition, decreases in arterial and venous BDFs before flicker provocation were identified in individuals with increased LDL-C levels. High LDL-C levels also negatively influenced the overall retinal venous DA during flicker.

In our study, and in line with previous research (Bolton-Smith et al. 1991; Arsenault et al. 2009), men exhibited higher BP and FRS as well as lower total CHOL and HDL-C compared

to age-matched women. Nevertheless, despite the observed differences there were no significant influences of these parameters on retinal vessel reactivity in either gender group.

We also observed lower levels of blood GSSG in women when compared to age-matched men. While a number of studies have revealed a relationship between redox balance and sex hormone patterns (Capel et al. 1981; Massafra et al. 1998; Diaz-Flores et al. 1999; Massafra et al. 2000; Serviddio et al. 2002; Browne et al. 2008; Cornelli et al. 2013), clinical reports of gender differences in glutathione levels are inconsistent with some studies reporting higher levels of GSH in men (Flagg et al. 1993) and others reporting no gender differences in plasma or blood glutathione levels (Michelet et al. 1995). These discrepancies may possibly arise due to variations in assay techniques. Our previous reports also reveal some degree of variability (Gherghel et al. 2005; Gherghel et al. 2013; Qin et al. 2013) however, with the methodology used in our own research being identical, these inconsistencies could also be attributed to other factors such as age. Indeed, the present study included a slightly younger group than our previous ones (Gherghel et al. 2005; Qin et al. 2013). Nevertheless, taken together, these observations suggest that gender might have a less consistent effect on blood glutathione levels than other confounding variables (Gherghel et al. 2013). Moreover, there were no differences with regards to retinal microvascular function parameters between men and women included in our study.

In the present study, we report for the first time a link between retinal arterial dilation during stress ($Slope_{AD}$) and redox index. From a microvascular standpoint endothelial health and redox balance are associative attributes (Frisard et al. 2007; Salmon et al. 2010). Indeed, it is well known that excessive levels of reactive oxygen species (ROS) are linked to an impaired endothelium-dependent dilation as they seek to quench NO, while the anti-oxidative properties of GSH involve the scavenging of such free radicals (Ghigo et al. 1993; Griscavage et al. 1994; Prasad et al. 1999). Typically, a decrease in the ratio of circulating reduced glutathione (GSH) relative to its oxidized form (GSSG) or 'redox index' has been proposed as an indicator of oxidative imbalance (Blankenberg et al. 2003) and oxidative stress status (Jones 2006; Jones 2006; James et al. 2009). Therefore, a positive relationship between $Slope_{AD}$ and redox index, could indicate that in addition to other processes, antioxidant mechanisms support normal NO secretion during flicker provocation at the retinal microvascular level. This possibility is further emphasised by the other novel discovery of a negative relationship between blood GSH levels and $Slope_{AC}$. Indeed, thiol oxidations during interactions with ROS are associated with endothelial dysfunction and increased vasoconstriction (Yamada et al. 2013). This effect has been previously reported at the coronary (Yamada et al. 2013), renal (Touyz 2004), and cerebral levels (Ong et al. 2013). Similarly, retinal microcirculatory reactivity also seems to be influenced by circulating levels of thiols. Interestingly, $Slope_{AC}$ also correlated with a more traditional risk factor, namely FRS. Few other studies have also observed similar relationships between established cardiovascular risk scores such as FRS and other measures of vascular function including peripheral arterial tonometry (Rubinshtein et al. 2010), digital thermal monitoring (Ahmadi et al. 2011), and whole-body MR angiography (Lehrke et al. 2009). Additionally, retinal vascular calibres have been shown to be independently associated with risk factor variables constituted in the FRS such as age, BP, HDL-C, and LDL-C (von Hanno et al. 2014) which are in turn associated with ocular pathologies of vascular consequence such as age-related macular degeneration (AMD) (Erke et al. 2014). More recently, semi-automated methods for quantitative assessment of the retinal vasculature show promise and may contribute additional value to morphological assessments (Schuster et al. 2014). In the present study, however, we have demonstrated not only a quantitative relationship between retinal vascular function parameters and systemic risk scores, but more specifically, a functional one based on the speed and amount of vascular reactivity defined by slope in the vascular response curves. This relationship characterizes the dynamic responsiveness of any vascular bed to increased demand, a parameter that determines each person's vascular reserve. This observation is particularly important since most of the efforts to prevent CVD are currently

focused on modifying population risk factors that often either over- or underestimate individualized risk (Cohn 2013). The assessment of the dynamic retinal vascular response to flicker represents a non-invasive way to determine the individual's functional vascular capacity and, therefore, the individual's likelihood to develop disease or to respond to vasoactive therapies currently used in treating CVD. Our observations could, therefore, suggest that individual vascular response profiles when used alongside traditional risk factors might improve the accuracy of CVD prediction (Akiyama et al. 2012; Matsuzawa et al. 2013).

Additional results of the present study also disclosed a negative relationship between LDL-C and arterial and venous BDF. LDLs are particularly susceptible to oxidative modifications and oxidized low-density lipoproteins (ox-LDLs) can inhibit the synthesis and release of endothelial nitric oxide synthase (eNOS) resulting in the decreased bioavailability of NO (Rosendorff 2002), as well as the increased expression of endothelin-1 (ET-1) and adhesion molecules (Ou et al. 2010; Itabe et al. 2011); all of which further contribute to the down-regulation of local retinal vascular tone and vessel stiffness (Laurent et al. 2005). On this basis, it could be hypothesized that the oxidative modification of LDL-C maybe associated with changes in the elastic properties of the vessel wall, reflected at the retinal vascular level as decreases in spontaneous variations of vessel diameter during normal resting conditions (Nagel & Vilser 2004). Indeed, ox-LDLs have been shown to impair vasomotor function of the coronary microcirculation (Hein & Kuo 1998). Moreover, LDL-C apheresis has been shown to improve endothelium-dependent vasodilation in hypercholesterolemic patients (Tamai et al. 1997), and to positively influence myocardial (Mellwig et al. 1998), cerebral (Rubba et al. 1993), and retinal (Terai et al. 2011) blood flow. A negative influence of LDL-C on the overall venous DA was also observed. Since retinal veins typically incite a more passive regulatory contribution to increases in blood flow (Kotliar et al. 2004), whether the overall decreases in DA may reflect the reconciliation of alterations in arterial outflow to the venous side via downstream autoregulatory mechanisms is unclear at present.

In our groups no correlations between measures of systemic macrovascular integrity and retinal microvascular function abnormalities were found. Interestingly, this finding is in concordance with that of a number of recent studies which have similarly demonstrated no direct correlation between anomalies identified at the macro- and micro-vascular levels in various disease states (Arosio et al. 2007; Heitmar et al. 2011; Sandoo et al. 2011). In addition, while C-IMT is a marker for structural abnormalities our measured retinal vascular parameters assess function which is affected first in the course of CVD. It is possible that in individuals with higher CVD risk than those included in the present study a positive correlation between anomalies identified at the macro- and micro-vascular levels would become apparent. Nevertheless, early identification of individuals at risk for CVD should be done when the disease is subclinical and well before structural changes begin to appear. Indeed, vascular changes in various beds appear to have common determinants since retinal vascular calibres have been shown to be associated with renal function in apparently healthy subjects (Daien et al. 2013). With this in mind, the direct relationship observed between systemic anti-oxidative defence capacity, plasma markers for CVD risk, and retinal dynamic vascular responses in apparently healthy individuals points toward more complex mechanisms that regulate the vascular response to stress at this level. In addition, it shows that assessing the retinal microvascular response in individuals free of overt vascular disease, but with a certain degree of CVD risk that is either age-related or due to other variables included in the FRS, could be a very sensitive indicator of each individual's specific risk. As molecular and imaging biomarkers drive the shift towards personalized medicine, retinal vessel reactivity can be used for profiling individualized vascular risk by providing an integrated and dynamic analysis of vascular function as a variable specific for each individual and, therefore, to be used in prediction, prevention, and personalised intervention.

Conflict of Interest

Conflict of interest: none declared

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Tables

Table 1. Summary of clinical characteristics

Variable	Mean (SD) Men [n=54]	Mean (SD) Women [n=48]	p-value
Age [years]	52 (8)	53 (7)	0.271
SBP [mmHg]	124 (14)	120 (15)	0.149
DBP [mmHg]	80 (10)	73 (11)	0.002*
HR [bpm]	65 (9)	64 (7)	0.495
IOP	14 (3)	14 (2)	0.904
BMI [kg/m2]	27.05 (4.17)	25.56 (3.89)	0.070
TG [mmol/L]	1.22 (0.55)	1.06 (0.34)	0.088
CHOL [mmol/L]	4.47 (0.80)	5.05 (0.96)	0.001*
HDL-C [mmol/L]	1.18 (0.36)	1.49 (0.37)	<0.001*
LDL-C [mmol/L]	2.74 (0.79)	3.07 (0.97)	0.056
C-IMT [cm]	0.07 (0.01)	0.06 (0.01)	0.064
FRS [%]	9.46 (5.94)	5.59 (4.35)	<0.001*
t-GSH [µmol/L]	1117 (772)	935 (643)	0.200
GSSG [µmol/L]	85 (73)	61 (48)	0.030*
GSH [µmol/L]	935 (713)	813 (586)	0.353
Redox index [GSH: GSSG]	15 (11)	20 (22)	0.140

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; IOP, intraocular pressure; BMI, body mass index; TG, triglycerides; CHOL, total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; C-IMT, carotid intima-media thickness; FRS, 10-year Framingham risk score for cardiovascular disease; t-GSH, total glutathione; GSSG, oxidized glutathione; GSH, reduced glutathione. *t*-test *p<0.05 was considered significant.

Table 2. Summary of retinal vascular function parameters as assessed by DVA

	Mean (SD)		p-value
	Men [n=54]	**Women [n=48]**	
Arteries:			
BDF	5.45 (2.14)	6.08 (3.63)	0.282
DA	8.05 (3.65)	7.77 (3.90)	0.704
MD [%]	5.15 (2.42)	4.71 (2.69)	0.385
tMD [seconds]	19 (6)	20 (8)	0.216
tMC [seconds]	28 (8)	27 (10)	0.767
$Slope_{AD}$	0.37 (0.24)	0.34 (0.25)	0.567
$Slope_{AC}$	-0.25 (0.11)	-0.31 (0.20)	0.060
$Slope_{AC}$, NU	1.15 (0.54)	1.02 (0.52)	0.195
Veins:			
BDF	4.38 (1.77)	4.30 (2.26)	0.866
DA	6.92 (2.93)	6.89 (2.94)	0.971
MD [%]	7.54 (5.07)	5.85 (3.18)	0.060
tMD [seconds]	20 (5)	22 (6)	0.099
tMC [seconds]	31 (9)	30 (9)	0.587
$Slope_{VD}$	0.32 (0.16)	0.49 (0.14)	0.408
$Slope_{VC}$	-0.23 (0.14)	-0.24 (0.13)	0.135
$Slope_{VC,}$ NU	1.49 (0.74)	1.36 (0.61)	0.365

Abbreviations: BDF, baseline diameter fluctuation (maximal range of baseline diameter measurements); DA, dilation amplitude (maximum diameter [MD] – minimum diameter [MC]); MD [%], percentage MD relative to baseline diameter; tMD, time taken to reach MD; tMC; time taken to reach MC from MD; $Slope_{AD/VD}$, slope of arterial/venous dilation (MD – baseline diameter) / tMD); $Slope_{AC/VC}$, slope of arterial/venous constriction (MC-MD / tMC); NU: normalized units. *t*-test/ANCOVA $p<0.05$ was considered significant.

Figure Legends

Figure 1. Relationship between low-density lipoprotein levels on (A) retinal arterial baseline diameter fluctuation, (B) retinal venous baseline diameter fluctuation, and (C) venous dilation amplitude. Abbreviations: LDL-C, low-density lipoprotein cholesterol; BDF, baseline diameter fluctuation; DA, dilation amplitude.

Figure 2. Relationship between the retinal arterial dilation response slope and redox index. Abbreviations: $Slope_{AD}$, retinal arterial dilation response slope; redox index, ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) levels.

Figure 3. Relationship between the retinal arterial constriction response slope and (A) circulating reduced glutathione, (B) Framingham Risk Score. Abbreviations: $Slope_{AC,}$ retinal arterial constriction response slope; GSH, reduced glutathione; FRS, 10-year Framingham Risk Score estimate for cardiovascular disease. Note: normalized unit values used on y-axes.