BRITISH JOURNAL OF BIOMEDICAL SCIENCE
2018, VOL. 75, NO. 3, 116–124
https://doi.org/10.1080/09674845.2017.1420130

The relationship of systemic markers of haemostasis with retinal blood vessel responses in cardiovascular disease and/or diabetes

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ABSTRACT

Background: Hypercoagulability is a leading factor in diabetes and cardiovascular disease. Retinal vessel responses to flickering light are an important tool for assessing ocular function. We hypothesised a significant relationship between systemic markers of haemostasis and retinal vessel function.

Methods: Intra-ocular pressure and retinal microcirculation function were measured in 116 patients with diabetes and/or cardiovascular disease using unstimulated and stimulated arterial and venous retinal vessel responses to flickering light. Haemostasis was evaluated by platelet microparticles, soluble P selectin, and five functional markers of fibrin clot formation and lysis, hyperglycaemia by HbA1c.

Results: Intra-ocular pressure was linked to the rates of clot formation ($p = 0.006$) and clot dissolution ($p = 0.013$) whilst central retinal vein equivalent was linked to HbA1c ($p = 0.017$). In the first of three flickering light cycles only, arterial baseline diameter fluctuation was linked to the lag time to clot formation ($p = 0.017$), whilst maximum venous dilatation was linked to HbA1c ($p = 0.001$) and clot density ($p = 0.011$). HbA1c was linked to venous dilatation amplitude ($p = 0.003$). There were no significant links between any ocular index and any platelet index.

Conclusions: In addition to glycaemia, several haemostasis measures, but no measures of platelet activity, are linked to ocular and retinal blood vessel indices in patients with diabetes and/or cardiovascular disease. These associations may have pathophysiological significance.

ARTICLE HISTORY
Received 14 November 2017
Accepted 1 December 2017

KEYWORDS
Microcirculation; retinal vessels; diabetes; thrombosis; fibrinolysis

Introduction

The retinal circulation is a target for the disease process in diabetes, and its non-invasive assessment by methods such as fundus photography, video recording and tomography has led to the view that it has potential as a screening tool for cardiovascular disease such as myocardial infarction and stroke [1,2]. This concept has been supported through large population studies [3,4] that together demonstrate a relationship between certain indices of retinal vessel function and cardiovascular risk [5,6]. Individuals with good cardiovascular health (normal blood pressure, glycaemia and lipidaemia) are less likely to have signs of retinopathy such as dilated retinal venules and narrow retinal arterioles, both of which are associated with increased risk of stroke and coronary artery disease [7].

Whilst retinal vessel calibre measurements provide only static indices, dynamic measurements such as retinal vessel reactivity to flicker light provocation can provide further insight into retinal microcirculation function [8]. Several authors have demonstrated a link between measures of cardiovascular health and retinal vessel dynamics, such as prolonged reaction times in retinal arterial responses to flickering light and decreased vessel dilation to flickering light in patients with coronary artery disease, and which depends on the severity of the disease [9,10]. There is also evidence that retinal vessel reactivity to flickering light provocation is blunted in the presence of diabetes and in diabetic retinopathy, although the precise pathophysiological mechanism for this is unclear [11,12].

Disruption of haemostasis (either of platelets or the coagulation pathway) is a leading aetiology in the development of terminal thrombotic events in cardiovascular disease, with both diabetes and hyperglycaemia being described as prothrombotic [13,14]. The finer aspects of pathophysiology include alterations to the structure and function of the fibrin clot and excessive platelet activation generating potentially occlusive microthrombi [15–17]. Routine markers of coagulation (prothrombin time, activated partial thromboplastin time) do not inform on clot integrity or dissolution, whilst the routine platelet count tells us nothing of the function of this cell. Platelet

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activation can be assessed by plasma markers such as soluble P selectin and platelet microparticles (PMPs) [18,19]. These pathways are important in ophthalmology as retinal vessel thrombosis is a leading ocular pathology [20,21].

We therefore hypothesised that retinal arterial and venous static and flicker responses are linked to functional indices of thrombosis and haemostasis in a patients with diabetes and/or cardiovascular disease.

Materials and methods

We recruited 116 patients from out-patient clinics at a University Teaching Hospital. Inclusion criteria were history of cardiovascular disease (myocardial infarction, stroke, >50% stenosis of artery proven by transcutaneous angiography, artery bypass grafting, amputation) and/or diabetes (HbA1c > 55 mmol/mol and/or attendance at a diabetes clinic). Under these criteria 36 had diabetes alone, 43 had cardiovascular disease alone and 37 had both. Exclusion criteria were age <18 years, connective tissue disease, cancer, recent (<3 months) cardiovascular events such as myocardial infarction or stroke, recent (<3 months) surgery, and established ocular disease such as age-related macular degeneration. Ethical approval was obtained from West Birmingham Ethics Committee and Aston University Ethics Committee. Written informed consent was received from all individuals taking part in the study. The study was designed and conducted in accordance with the Declaration of Helsinki.

The ocular protocol was as follows. On the study day, all subjects refrained from consuming caffeinated products, chocolate, drinking alcohol and smoking. Intraocular pressure (IOP) was measured by contact tonometry (I-Care, Mainline Instruments Ltd, Birmingham, UK) and calculated as the mean of six consecutive readings. Dynamic and static retinal vessel assessment was determined after full pupil dilation was reached with 1% tropicamide (Chauvin Pharmaceuticals Ltd., Kingston, UK), digital fundus images and reactivity parameters of retinal blood arteries and veins were obtained by a retinal vessel analyser [RVA] (Imedos Systems, Jena, Germany)[10].

For static vessel analysis, black and white fundus images were obtained at a 30° angle with the optic nerve head centred, using an inbuilt Zeiss FF450plus fundus camera (Zeiss GmbH, Germany). Arterial and venous diameters provided an arterio-venous-ratio (AVR), central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) (Vesselmap software, Imedos Systems, Jena, Germany) [22,23]. AVR, CRAE and CRVE are standard ophthalmological indices used to describe the luminal diameter of retinal arteries and veins.

In the dynamic assessment that followed, retinal diameters were measured continuously at a sampling rate of 25 Hz. Retinal blood vessels were stimulated by flickering light provocation with a 12.5 Hz frequency [24]. A segment of the superior temporal retinal artery and vein (minimum 500 microns in length) was chosen at a distance of 1.5–2 disc diameters from the margins of the optic nerve head. Baseline diameter of both the artery and vein was recorded according to the standard RVA protocol [25] for 50 s and then followed by three cycles of 20 s flicker provocation with 80 s recovery each time. From the diameter recordings, the values for maximum dilation (MD), maximum constriction (MC), dilation amplitude (DA), arterial baseline corrected flicker response (BFR), arterial and venous reaction times (RT) to flicker provocation were calculated [26].

Blood samples were collected from an antecubital vein directly into Vacutainer tubes (Becton Dickinson, Oxford, UK) containing 0.5 ml 3.2% sodium citrate. For microparticle detection, platelet-poor plasma was obtained after 15 min centrifugation of citrated blood at 2,800 g and further centrifugation at 13,000 g for 2 min to remove residual cellular fragments to obtain platelet-free plasma as per ISTH guidelines [27]. Platelet free plasma was initially incubated for 30 min with 0.5 µg of biotinylated antihuman CD42b antibody (Abcam, Cambridge, UK). This was followed by a second incubation with 0.25 µg of StreptavidinAlexa Fluor647 nmRPhycoerythrin conjugate (Life Technology, Paisley, UK) for 30 min and then diluted with 990 µl filtered PBS (final dilution 1:100). Microparticle analysis was performed promptly using an Apogee A50 flow cytometer (Apogee Flow Systems, High Wycombe, UK). The size of the PMP was confirmed with polystyrene (110, 200, 500 nm and 1 µm diameter) and silica (300 and 880 nm) beads (Apogee Flow Systems, Hemel Hempstead, UK). Full details are available elsewhere [28,29]. Soluble P selectin (sPsel) was measured by commercial ELISA (R&D Systems, Abingdon, UK).

Thrombogenesis and fibrinolysis were assessed by a 96-well microtitre plate assay [16,30]. In the thrombogenesis assay, the formation of a fibrin clot over a 10-minute period is followed by the increase in optical density of the plasma as it becomes a clot, in a microtitre plate reader at 37 °C. Software from the plate reader determines the outcome data: the lag time from recalcification to the initiation of clot formation, the rate of clot formation, and the maximum optical density (a surrogate of clot integrity). In a separate microtitre plate, fibrinolysis is assessed by a fall in the optical density due to the action of extrinsic tissue plasminogen activator, giving the rate of clot dissolution (optical units/min) and the time for 50% of the clot to lyse (T50). HbA1c was measured in EDTA-anticoagulated whole blood using standard techniques by the Hospital Routine Pathology Laboratory. This determination was performed at the same venepuncture as provided blood for the research indices.

We tested the hypothesis that markers of platelet activation (as defined by sPsel and PMPs), thrombogenesis (Lag time, rate of clot formation, maximum optical

Table 1. Clinical data, medication and demographics.

Feature	Data	Feature	Data
Demographics			
Age (years)	64.3 (9.9)	Sex (male/female)	86/30
Weight (kg)	86.6 (16.6)		
Clinical			
Body mass index (kg/m^2)	29.3 (5.5)	SBP (mm Hg)	126 (16)
DBP (mm Hg)	73 (11)	Heart rate (bpm)	72 (13)
Co-morbidities			
Coronary artery disease (*n*, %)	61, 52.6%	Peripheral artery disease (*n*, %)	9, 7.7%
Cerebrovascular disease (*n*, %)	11, 9.5%	Diabetes (*n*, %)	73, 62.9%
Medications			
Calcium channel blocker (*n*, %)	47, 40.5%	ACEI/ARB (*n*, %)	84, 72.4%
Lipid-lowering (*n*, %)	103, 88.8%	Aspirin (*n*, %)	76, 67.8%
Clopidogrel (*n*, %)	15, 12.9%	Nitrate (*n*, %)	22, 19.0%
Oral anticoagulant (*n*, %)	16, 13.8%	Beta-blocker (*n*, %)	51, 44.0%
Diuretic (*n*, %)	45, 38.8%	Thyroxine (*n*, %)	15, 12.9%
Metformin (*n*, %)	47, 40.5%	Sulphonylurea (*n*, %)	14, 12.1%
DPP-4 inhibitor (*n*, %)	14, 12.1%	Insulin (*n*, %)	29, 25.0%
GLP-1 agonist (*n*, %)	8, 6.9%	Piaglitazone (*n*, %)	6, 5.2%

Notes: SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; HbA1C: glycated haemoglobin; ACEI: angiotensin-converting-enzyme inhibitor; ARB: Angiotensin receptor blockers; DPP-4 = dipeptidyl peptidase-4 ('gliptins'); GLP-1 = glucagon-like peptide-1 (exenatide, liraglutide). Data presented as mean with standard deviation, or as number of subjects and percentage.

density), fibrinolysis (rate of clot dissolution) and glycaemia (HbA1c) are linked to retinal vessel function in a multi-variate linear regression analysis taking each ocular index as the dependent variable and the eight research indices as independent variables, and in a general linear model. Indices with a non-normal distribution were log transformed for multi-variate analyses. A sample size of at least 10 is required for each independent variable, of which we have 8, thus calling for a total sample size of at least 80 patients. However, in view of the possible likelihood of an inter-relationship between the several of the ocular and research indices, and in order to obtain greater confidence, we decided to over-recruit by at least a third (i.e. to at least 107 patients), eventually recruiting 116 patients. Continuously variable data are presented as mean and standard deviation or as median and interquartile range as distribution demands. Categorical data are presented as number and percentage. Correlation were sought (when justified) by Pearson's method. In view of multiple analyses, significance was assumed at $p < 0.02$ [35]. Analyses were performed on Minitab version 17 (Minitab, Coventry, UK).

Table 2. Haemostasis, and resting ocular and retinal vessel indices.

Research index	Data
Platelet markers	
Platelet microparticles (x10^3/μL)	10.6 (0.9–54.9)
Soluble P selectin (ng/mL)	16 (13–24.5)
Coagulation markers	
Lag time to start of thrombus formation (min)	5.5 (4.8–6.8)
Rate of clot formation (Units/s)	12.8 (9.1–17.2)
Maximum optical density (Units)	0.36 (0.12)
Fibrinolysis markers	
Rate of clot dissolution (Units/s)	41.5 (17.1)
T50 (min)	3.5 (0.8)
Ocular indices	
Intra-ocular pressure (mm Hg)	14.1 (2.5)
Central retinal artery equivalent (arbitrary units)	176 (17)
Central retinal vein equivalent (arbitrary units)	212 (19)
Artery/vein ratio	0.83 (0.09)
Artery diameter (μm)	113 (19)
Vein diameter (μm)	141 (19)

Notes: T50 = time for 50% of the clot to lyse. Data presented as mean (SD) or median (lower quartile–upper quartile).

Results

Tables 1–3 show the clinical, demographic, haemostasis and ocular indices of the 116 patients. Median duration of disease in the 73 diabetics was 10 years (interquartile range 4.5–16.5 years). For each ocular index in Tables 2 and 3, we first performed a univariate analysis of all research eight indices, selecting only those with $p < 0.02$ for multivariate analysis. This analysis found that intra-ocular pressure was linked to the thrombogenesis index rate of clot formation in univariate analysis, and this was retained in multivariate analysis (Table 4). Intra-ocular pressure was also linked to the thrombolysis index rate of clot dissolution in univariate analysis, and in multivariate analysis this too was retained. The rate of clot formation correlated with the rate of clot dissolution ($r = 0.34$, $p = 0.001$). The thrombolysis index T50 (time for 50% of the clot to lyse) was linked to intra-ocular pressure in univariate but not in multivariate analysis.

Central retinal vein equivalent was linked to the HbA1c in univariate and multivariate analyses. Arterial baseline diameter fluctuation after the first flicker cycle was linked to the lag time to clot formation in univariate analysis but not in multivariate analysis. Similarly, HbA1c and clot density (as maximum optical density) were linked in multivariate analysis to maximum venous dilatation. HbA1c and clot density were also linked to venous dilatation amplitude, but in multivariate analysis, only the link with HbA1c remained significant. The parallels in these latter analyses may be part-explained by the correlation coefficient between venous maximum dilatation and dilation amplitude of 0.73 ($p < 0.001$), although the HbA1c and maximum optical density were not linked ($r = 0.17$, $p = 0.103$). There were no significant links in subsequent flicker cycles. There were no relationships between either platelet index and any ocular index. Results were confirmed in the general linear model.

Discussion

The eye presents a unique opportunity to non-invasively assess the microcirculation, and may be useful in predicting those at risk of cardiovascular disease [31]. A further development in ocular pathology is the recognition of the value of retinal vessel responses to flickering light in

Table 3. Retinal vessel responses in the flicker cycles.

	Flicker cycle 1	Flicker cycle 2	Flicker cycle 3	Averaged flicker response
Arterial responses				
Maximum dilatation (%)	3.8 (3.5)	3.6 (3.2)	4.0 (3.4)	2.8 (2.1)
Maximum constriction (%)	−2.2 (1.9)	−2.4 (1.9)	−2.1 (1.8)	−1.9 (1.4)
Dilatation amplitude (%)	5.1 (3.3–7.7)	5.0 (3.1–7.7)	5.1 (3.2–7.5)	4.0 (2.6–6.4)
Reaction time (s)	18 (10–22)	18 (13–26)	19 (14–31)	18 (12–24)
Venous responses				
Maximum dilatation (%)	4.4 (2.1)	4.8 (2.5)	4.7 (3.0)	4.3 (2.0)
Maximum constriction (%)	−1.3 (1.8)	−1.1 (2.0)	−1.1 (2.4)	−0.8 (1.3)
Dilatation amplitude (%)	5.0 (3.9–7.3)	5.4 (4.1–7.4)	5.2 (3.6–7.3)	4.6 (3.5–6.2)
Reaction time (s)	20 (17–23)	20 (15–23)	20 (15–23)	20.4 (6.7)

Notes: Summarised Retinal Vessel Calibres and software generated vessel reactivity parameters and IMEDOS software generated arterial and venous responses to flicker. Data presented as mean (SD) or median (lower quartile-upper quartile).

Table 4. Univariate and multivariate links between ocular indices and laboratory indices.

Ocular index	Laboratory index	P value in univariate analysis[b]	P value in multivariate analysis[c]
Intra-ocular pressure	Rate of clot formation	<0.001	0.006
	Rate of clot dissolution	0.001	0.013
	T50	0.032	Not selected
Central retinal vein equivalent	HbA1c	0.017	0.022
Arterial baseline diameter fluctuation[a]	Lag time to clot formation	0.011	0.418
Venous maximum dilatation[a]	HbA1c	0.001	0.001
	Maximum optical density	0.007	0.011
Venous dilatation amplitude[a]	HbA1c	0.001	0.003
	Maximum optical density	0.014	0.071

[a]In the first flickering light cycle. T50 = time for 50% of the fibrin clot to lyse.
[b]Where all eight laboratory indices are entered: associations that are not significant are not shown.
[c]Where only those significant at $p < 0.02$ in the univariate analysis are entered.

diabetes and cardiovascular disease [32–34]. A potential pathophysiological process to explain these abnormal retinal vessel responses is an imbalance in haemostasis leading to a tendency to thrombosis [13–15]. We tested the hypothesis that retinal vessel responses in patients with diabetes and/or cardiovascular disease are linked to variability in haemostasis, factoring in glycaemia as a reference pathological process.

Unsurprisingly, given the established pathology of this risk factor marker [35], we found that HbA1c is linked to several venous retinal vessel indices – the static central retinal vein equivalence (diameter), and the dynamic venous maximum dilatation and dilatation amplitude in response to flickering light. None of the arterial indices was linked to HbA1c, and there were no significant links in the second and third flicker cycles. These findings contribute to the general debate of the role of hyperglycaemia and retinal vessel responses in diabetes, and may be partially explained by a damaging effect of hyperglycaemia on the endothelium [10–12,36].

Parallel analyses also revealed links with haemostasis. Intra-ocular pressure was linked to both the rate of clot formation and to the rate of clot dissolution. The consequences of these links for pathophysiology are unclear and do not directly determine causality (i.e. increased intra-ocular pressure is not necessarily the consequence of abnormal clot formation or clot dissolution). The same conclusion must be drawn with the relationship between the lag time to clot formation and baseline arterial diameter fluctuation, and that between the maximum optical density (reflecting clot density) and venous responses to flickering light – the maximum dilatation and the dilatation amplitude. It is again unclear if these changes have pathophysiological consequences, but it is tempting to speculate that abnormal haemostasis may play a part (if only minor) in artery and vein responses to the flickering light. This may operate via microthrombi adhering to, and so influencing, the function of retinal vessel endothelium, and/or by microthrombi occluding micro-vessels. Similarly, it could be argued that increased HbA1c, the gold standard marker of hyperglycaemia and so of a risk of, or actual, cardiovascular disease, suppresses the responses of retinal veins to flickering light by an action on the endothelium [37] and potentially on haemostasis in these patents [38]. A further caveat is the presumption that blood obtained from an ante-cubital vein behaves as it does in the ocular microcirculation, which may not be the case. We note that, against expectation [39,40], neither platelet indices seem to have a role in ocular or retinal vessel function, possibly because of the use of anti-platelet drugs by all patients with cardiovascular disease.

We acknowledge the limitation of multiple analyses, and that many of these are likely to be physiologically related and so we may be a risk of false positives. However, we feel this is countered by the large sample size, our more stringent p values, and that we have not over-interpreted our data. Our data may also be limited by possible effects of various systemic medications being taken by the patients, and that we recruited from a well-motivated group that may contribute to a better than expected haemostasis compared to poorly motivated patients on different medications. Nevertheless, our population is fully clinical in that all were being seen

in a secondary care setting for diabetes and/or cardiovascular disease, and so represent the full spectrum of diabetic atherosclerosis. It is also likely that many patients with diabetes would have asymptomatic cardiovascular disease.

We suggest our data represent an advance in biomedical science because it shows that thrombogenesis and fibrinolysis may influence intra-ocular pressure and retinal vessel function in patients with diabetes and/or cardiovascular disease.

Summary table

What is known about this subject

- Cardiovascular disease is the leading cause of morbidity and mortality linked with diabetes
- The retinal circulation is one of the targets for the disease process, the leading aetiology in diabetic cardiovascular disease is thrombosis
- Retinal vessel responses to flicking light are impaired in diabetes and cardiovascular disease, but the mechanism is unknown

What this paper adds

- Intra-ocular pressure is linked to clot formation and dissolution *in vitro*
- Abnormalities retinal vessel function in the first flickering light cycle were linked to indices of thrombogenesis and hypergylcaemia: these were not evident in subsequent cycles

Acknowledgements

We thank our colleagues Drs R Ryder, C Varma, P De and J Khan for help in recruitment.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by a Hans and Gertrude Hirsch Small grant from Fight for Sight (www.fightforsight.org.uk).

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