

Cardiovascular Topics

Delayed retinal vein recovery responses indicate both non-adaptation to stress as well as increased risk for stroke: the SABPA study

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Abstract

Objectives: Low or high sympatho–adrenal–medullary axis (SAM) and hypothalamic–pituitary–adrenal axis (HPA) dysregulation reflect chronic stress. Retinal vessel dynamics may relate to SAM, HPA activity and stroke risk. Our objectives were therefore to assess the relationships between retinal vessel, SAM and HPA responses, and to determine stroke risk.

Methods: A prospective bi-ethnic gender cohort ($n = 275$, 45 ± 9 years) was included. Urine/serum/saliva samples for SAM [norepinephrine:creatinine ratio (u-NE)] and HPA [adrenocorticotrophic hormone (ACTH), cortisol] were obtained at baseline, three-year follow up and upon flicker light-induced provocation. Diastolic ocular perfusion pressure was measured as a marker of hypo-perfusion. Retinal arterial narrowing and venous widening calibres were quantified from digital images in the mydriatic eye. A validated stress and stroke risk score was applied.

Results: An interaction term was fitted for venous dilation in u-NE tertiles ($p \leq 0.05$) and not in u-NE median/quartiles/quintiles. Independent of race or gender, tertile 1 (low u-NE) had a 112% increase in u-NE, decreases in cortisol, and no changes in ACTH over three years (positive feedback). Tertile 3 (high u-NE) contradictorily had decreases in u-NE and cortisol, and increases in ACTH (negative feedback). In tertile 1, reduced arterial dilation, and faster arterial vasoconstriction and narrowing were related to higher SAM activity and hypo-perfusion ($p \leq 0.05$), whereas delayed venous dilation, recovery and widening were related to cortisol hypo-secretion ($p \leq 0.05$). In tertile 1, delayed venous recovery responses predicted stress and stroke risk [odds ratio 4.8 (1.2–19.6); $p = 0.03$]. These associations were not found in u-NE tertiles 2 and 3.

Conclusions: In response to low norepinephrine, a reflex increase in SAM activity occurred, enhancing arterial vasoconstriction and hypo-perfusion. Concomitant HPA dysregu-

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lation attenuated retinal vein vasoactivity and tone, reflecting delayed vein recovery responses and non-adaptation to stress. These constrained vein recovery responses are indicative of increased chronic stress and stroke risk.

Keywords: retina, stress, norepinephrine, HPA, hypo-perfusion, stroke

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The retina shares embryonic origins with the brain, with similar anatomy and blood-barrier physiology. The retina is therefore of particular interest as a marker of cerebrovascular¹ and neurodegenerative diseases.² Local perfusion mechanisms are also similar as an increase in neuronal activity within the brain evokes local increases in blood flow or functional hyperaemia.³ Functional hyperaemia ensures that active neurons receive sufficient oxygen and nutrients to maintain tissue functionality in the blood–retinal barrier (BRB).⁴

The inner BRB is formed by specialised retinal microvessels, surrounding pericytes and astrocyte end-feet to form a functional neurovascular unit (coupling).^{4,6} Astrocyte end-feet envelope arterioles and capillaries, covering the vascular surface, and directly interact or communicate with vascular smooth muscle cells and pericytes⁵ (Fig. 1). Retinal vessels, therefore, offer an easily accessible view of the vasculature that responds to flicker light-induced provocation (FLIP), and which might reflect emotional stress pathology and stroke risk.

It is well-known that chronic stress facilitates the release of neurotransmitters and hormones such as norepinephrine, adrenocorticotrophin (ACTH) and cortisol via key neuroendocrine signalling pathways, namely the sympathetic–adrenal–medulla axis (SAM) and hypothalamic–pituitary–adrenal axis (HPA).⁵⁻¹² Dysregulation of the SAM and HPA hormones are related to structural degeneration in the hippocampus and prefrontal cortex, while impaired functioning reflect cerebrovascular perfusion deficits.¹³ Stress, facilitating higher sympathetic activity and metabolic demands, promotes active transport of norepinephrine in the central nervous system (CNS),^{13,14} increasing local blood flow or functional hyperaemia³ and risk for ischaemic stroke.^{15,16}

Stress hormones released from astrocyte end-feet may, therefore, have a direct effect on retinal vessel dilation or constriction, as norepinephrine is an effective stimulator of adenylate cyclase, which compromises integrity of the BRB.⁵ Similar cerebral neurovascular mechanisms in the brain–retina axis may further underscore the interrelationship between psychopathology and neurodegenerative disease.^{17,18} Indeed, Alzheimer's disease and depression as neurodegenerative diseases have recently been associated with delayed retinal vessel dilation upon FLIP, reflecting increased sympathetic tone.¹⁷

We have previously reported that the stroke risk markers, retinal artery narrowing and vein widening,¹⁸ were related to depressed heart-rate variability (HRV)¹⁹ and stroke risk in a bi-ethnic cohort.²⁰ It is therefore feasible to link neurodegenerative disease and psychopathology assessments in

the BRB, as the inner neural retinal layers and cell components express adrenergic receptors (AR) namely α_{1a} -AR,¹⁰ α_{2a} -AR^{21,22} as well as glucocorticoid receptors (GCR).² α_{1a} -AR increases norepinephrine release and vasoconstriction, whereas α_{2a} -AR inhibits norepinephrine release to protect ganglion cells in the optic nerve head (Fig. 1).

Norepinephrine or adrenergic receptor-driven changes in retinal vessel dynamics and tone may reflect neuronal hyperactivity or adrenergic drive. The GCR protect retinal neurons by suppressing inflammation and inhibiting microglial cells to block the production of cytotoxic molecules.²³ Corticosteroid hormones control vascular smooth muscle tone by their permissive effects in potentiating vasoactive responses to catecholamines through GCR.²⁴ Flicker provocation, as acute mental stressor, may therefore reflect norepinephrine (SAM) and HPA's function on sensory processing via receptor activation or inhibition.

We previously observed that the cardiac and retinal microvasculature reflected depressed HRV and hypo-perfusion.^{18-20,25-27} Whether stress hormones will disturb retinal vessel responsiveness to increase the risk for stroke has yet to be determined. The aim of this study was therefore (1) to investigate temporal relationships between the retinal vasculature, SAM and HPA responses over three years and upon provocation, and (2) to determine stress and stroke risk.

Methods

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) prospective study was conducted from late summer until late autumn in 2008/9, and after three years, in 2011/12. A teachers' cohort (20–65 years), having similar socio-economic status, was included.²⁵ Baseline exclusion criteria were tympanum temperature $\geq 37.5^\circ\text{C}$, pregnancy and/or lactation, α - and/or β -blocker use, psychotropic substance use, as well as vaccination and/or blood donation three months prior to participation. Only participants who participated in both phases ($n = 359$) were included for the current investigation. Additional exclusions were poor-quality retinal vessel images and missing data ($n = 65$), stroke ($n = 1$), HIV infection ($n = 19$), and a user of central nervous stimulants ($n = 1$). The final participant sample comprised 273 individuals.

Participants were fully informed about the objectives and procedures prior to recruitment and provided written, informed consent. The study conformed to the Helsinki Declaration (2004) and was approved by the ethics review board of the North-West University, Potchefstroom campus (approval number NWU-0003607S6).

During the working week, 24-hour ambulatory blood pressure and ECG monitors (Cardiotens CE120[®], Meditech, Budapest, Hungary) were fitted to teachers at their school of employment at approximately 07:00. A 24-hour standardised diet plus 24-hour urine sampling commenced, after which participants resumed their normal daily activities. At 15:00, participants were transported to the North-West University for retinal vessel imaging and an overnight stay in a relaxed, well-controlled environment. For the remaining clinical measures each participant received his/her own room and was informed on the experimental set-up and sampling conditions to lessen anticipation stress. Hereafter, registered clinical psychologists

supervised the completion of the depression questionnaire²⁵ and participants were advised to go to bed at 22:00, fasting overnight.

The next morning, after the last 24-hour blood pressure (BP) recording at 07:00, the Cardiotsens CE120[®] apparatuses were disconnected. BP and two lead ECG time-domain HRV

analyses¹⁹ were done using the CardioVisions 1.19 personal edition software (Meditech, Budapest, Hungary). Anthropometric and total energy expenditure measures were performed according to standardised procedures. Hereafter, participants were in a semi-recumbent position for at least 30 minutes before saliva

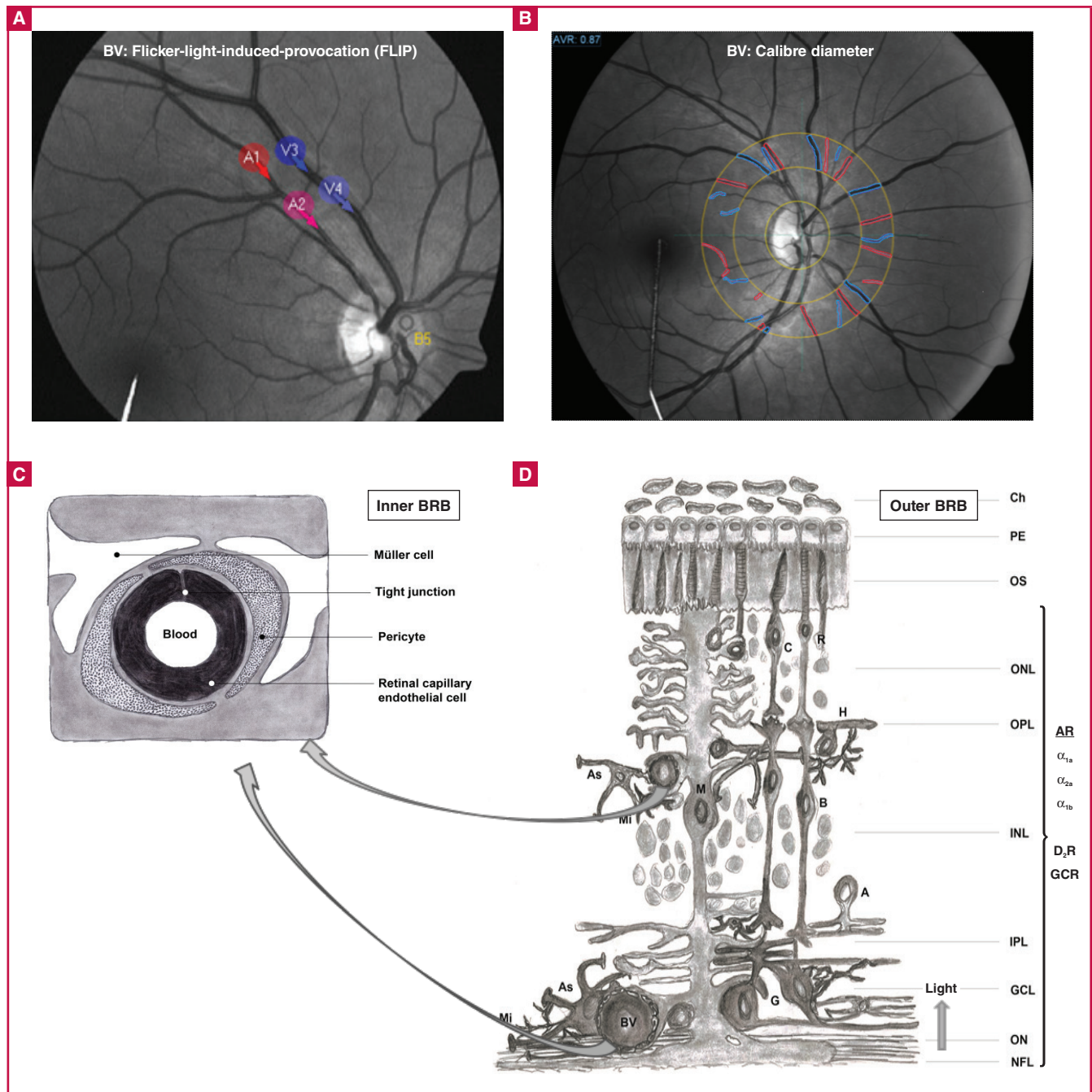


Fig. 1. Presenting retinal haemodynamic assessment sites and neurovascular coupling between glial cells and blood vessels (BV). A. The selected artery (A = red) and vein (V = blue) areas to determine BV responses upon flicker light-induced provocation. B. Retinal BV to determine arterial narrowing (red) and vein widening (blue). C, D. The blood vessel characteristics in the inner and outer blood–retinal barrier (BRB). The inner BRB (C) contains capillary endothelial cells and the outer BRB (D) contains pigment epithelial (PE) cells. D shows the inner and outer BRB retinal neural layers (bottom to top). NFL, optic nerve fibre layer; ON, optic nerve; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer with horizontal cells (H), bipolar cell dendrites (B), amacrine cells (A), astrocytes (As), microglia (Mi); Müller cells (M); ONL, outer nuclear layer with rods (R) and cones (C); OS, outer segment layers; PE, pigment epithelial cells; Ch, choroid; AR, adrenergic receptors in the OPL (α_{1a} -AR, α_{1b} -AR, α_{2a} -AR); D₂R, dopamine₂ receptors; GCR, glucocorticoid receptors (Malan *et al.*⁴ Adapted by Louise Malan).

and blood sampling. After all assessments, immediate available feedback was given in the privacy of their rooms.

Retinal vessel analyses considering arteriolar and venular calibres (hereafter referred to as arteries and veins) were done at follow up. One hour prior to measurements, no intake of food or caffeine-containing beverages, alcohol, smoking or exercise were allowed and this is well-described elsewhere.²⁵⁻²⁷ Analyses were performed in a well-controlled light- and temperature-regulated laboratory using the Retinal Vessel Analyser (Imedos Systems GmbH, Jena, Germany) with a Zeiss FF450^{plus} camera and the software Vessel-Map 2, Version 3.02.²⁸

Participants were introduced to the procedure and screened for acute anterior angle chamber glaucoma risk with a small light source by a trained registered nurse. Mydriasis was induced in the right eye of the participant by instilling a drop containing tropicamide 1% and benzalkonium chloride 0.01% (m/v). Retinal haemodynamic responses were assessed upon provocation (FLIP) by measuring the diameter of retinal arteries and veins continuously. The absolute vessel diameter of measured arterial and venous segments was calculated individually as a median value before the first flickering. Three single curves were obtained during each flicker cycle in each subject and consisted of (1) 30 seconds of baseline before the flicker application, (2) 20 seconds of flicker application, and (3) 80 seconds thereafter were recalculated as a percentage of their baseline values and averaged to one.¹⁷

An innovative approach was applied to assess SAM and HPA activity by obtaining time-domain HRV and salivary α -amylase

and cortisol responses upon provocation. Salivary sampling was done prior to and after provocation (changes from baseline were calculated in μM and in %). A salivette cotton swab was placed in the mouth and passive-drooling sampling was done for one to two minutes (Salimetrics[®]). Samples were immediately placed on ice and frozen at -80°C until analysis. The tweezers used to place the saliva cotton swab (Sarstedt Inc, Leicester, UK) in the mouth was kept in a 0.5% chlorhexidine gluconate solution and rinsed with distilled water before sampling.

Retinal artery and vein calibres were measured as monochrome images to indicate arterial narrowing and vein widening (indicative of stroke risk).^{4,16} First-order vessel branches were manually selected in a measuring zone located between 0.5 and 1.0 optic disc diameters from the margin or the optic disc (Fig. 1B). Upon selection of the vessel, software automatically delineated the vessels' measuring area. Identification of vessels was done by two experienced scientists who had to agree on the vessel type before selection. Automated software calculations, based on the Knudtson revision of the Parr-Hubbard formulae, determined estimates from the six largest arteries and veins and were expressed as measuring units (MU). One MU is equivalent to 1 μm when the dimensions of the eye being examined correspond to those of the normal Gullstrand eye. Reproducibility was computed for a randomly selected cohort with a correlation coefficient of 0.84.

Microvasculature perfusion as a retinal/cerebral hypoperfusion risk marker¹⁸ was measured by instilling a local

Table 1. Dependent sample *t*-tests presenting clinical risk marker changes over a three-year period by norepinephrine:creatinine (u-NE nmol/l:mmol/l) tertile status

	<i>u</i> -NE tertile 1 median (min-max): 8.74 (1.05-14.77) (n = 93)		<i>u</i> -NE tertile 2 median (min-max): 21.23 (15.05-28.62) (n = 91)		<i>u</i> -NE tertile 3 median (min-max): 40.62 (28.69-113.63) (n = 91)	
	Baseline/ follow up	Difference (95% CI)	Baseline/ follow up	Difference (95% CI)	Baseline/ follow-up	Difference (95% CI)
Whites, n (%)	54 (58.1)		44 (48.4)		68 (74.7)	
Men, n (%)	58 (64)		50 (54)		29 (32)	
Age \pm SD (years)	43.2 \pm 8.9		45.3 \pm 9.5		47.4 \pm 8.9	
Potential vasculature risk factors						
Depression	6.7/6.2	-0.8 (-1.9, 0.3)	7.4/7.1	-0.3 (-1.4, 0.7)	7.5/7.1	-0.6 (-1.5, 0.3)
WC, cm	92.7/98.0	7.2 (3.8, 6.8)**	94.1/96.2	2.1 (0.8, 3.3)**	93.1/95.8	2.7 (1.0, 4.4)**
Physical activity, kcal/24 h	3182.2/3622.2	440.1 (-135.0, 1015.1)	2805.8/3385.6	579.8 (278.7, 880.8)**	2866.8/3195.7	328.8 (92.9, 564.7)**
Cotinine, ng/ml	26.3/21.9	-4.4 (-10.5, 1.7)	24.6/34.6	10.0 (0.2, 19.9)*	32.1/32.8	0.7 (-12.8, 14.2)
γ -GT, u/l	38.1/37.4	-0.7 (-8.5, 7.2)	49.0/41.6	-7.4 (-16.8, 1.9)	35.2/30.5	-0.4 (-10.0, 0.6)
CRP, mg/l	4.5/5.0	0.5 (-2.7, 3.7)	5.6/4.1	-1.6 (-2.9, -0.3)*	5.0/3.3	-1.7 (-2.6, -0.8)**
HDL-C, mmol/l	1.1/0.9	-0.1 (-0.2, -0.1)**	1.2/1.1	-0.2 (-0.2, -0.1)**	1.2/1.1	-0.1 (-0.2, -0.1)**
TG, mmol/l	1.3/1.3	0.01 (-0.1, 0.1)	1.4/1.3	-0.1 (-0.4, 0.2)	1.2/1.2	-0.1 (-0.2, 0.0)
HbA _{1c} , %	5.7/5.8	0.1 (-0.1, 0.2)	5.8/5.8	0.0 (-0.1, 0.1)	5.7/5.8	0.1 (-0.04, 0.3)
Stress hormone markers						
NE (ng/ml)	23.9/45.4	21.5 (14.7, 28.3)**	41.6/58.2	16.6 (6.4, 26.9)**	66.7/65.3	-1.4 (-13.5, -1.7)
Creatinine, mmol/l	16.8/17.9	1.1 (1.8, 3.9)	11.4/16.6	5.1 (2.5, 7.7)**	8.4/12.4	4.0 (2.1, 5.9)**
u-NE, nmol/l:mmol/l	8.6/18.2	9.6 (6.5, 12.6)**	21.3/24.1	2.7 (-0.8, 6.1)	49.7/40.5	-9.2 (-15.6, -2.8)**
Cortisol, nmol/l	400.1/245.1	-154.4 (-187.9, -120.9)*	374.4/243.7	-130.7 (-164.1, -97.3)**	357.6/213.2	-145.9 (-178.1, -113.7)**
ACTH, pg/ml	19.0/18.0	-0.97 (-3.3, 1.3)	18.6/18.8	0.27 (-2.6, 3.1)	15.6/18.4	3.2 (1.3, 5.2)**
Time-domain heart-rate variability						
SDNN (ms)	239.4/134.9	-104.5 (-293.7, 84.8)	127.7/120.1	-7.8 (-15.5, -0.1)*	132.0/127.8	-4.2 (-12.9, 4.4)
Blood pressure						
24-h SBP, mmHg	128/128	0.2 (-3, 2)	128/130	2.0 (0.0, 4.0)*	127/126	-1.5 (-4, 1)
24-h DBP, mmHg	79/79	0.0 (-2, 1)	80/80	0.3 (-1, 2)	80/77	-2 (-4, -1)**

Depression, Patient Health Questionnaire-9 (DSM-IV criteria); WC, waist circumference; γ -GT, gamma-glutamyl transferase; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; HbA_{1c}, glycated haemoglobin; ACTH, adrenocorticotropic hormone; SDNN, standard deviation of the normal-to-normal (NN) intervals between adjacent QRS complexes, which equal the square-root of variance. Values are presented as arithmetic mean at baseline/follow up as well as the difference over three years (95% CI). *p*-values obtained from dependent sample *t*-tests; **p* \leq 0.05; ***p* \leq 0.001.

anaesthetic drop (Novasine Wander 0.4% Novartis) in the right eye to measure intra-ocular pressure (IOP) (Tono-Pen Avia Applanation Tonometer; Reichert 7-0908, ISO 9001, New York, USA). Diastolic ocular perfusion pressure (DOPP) was calculated prior to FLIP: [diastolic blood pressure (DBP)–IOP mmHg]. Hypertensive/diabetic retinopathy was diagnosed by a registered ophthalmologist.

For SAM sampling, overnight eight-hour urine sampling was performed at baseline with 24-hour sampling at follow up. The sampling periods of eight and 24 hours compare favourably for detection of norepinephrine in urine.²⁹ At the three-year follow up, participants began and ended sampling with an empty bladder on day one and commenced with a 24-hour standardised dinner. Urine was collected for the next 24 hours in a three-litre container, washed with 9 ml of 20% HCl (UriSet24, Sarstedt®, Nümbrecht, Germany). Urine samples were stored at –80°C until analysis, which occurred within one year from collection.

The 3-Cat Urine ELISA Fast Track kits (SKU: BA E-6600, LDN, Nordhorn, Germany) were used where a standard range of 0.5–1 000 ng/ml was reported. Mean levels of norepinephrine 42 ng/ml (standard error ± 4.4) at baseline with 49 ng/ml (standard error ± 4.6) at follow up were apparent in the SABPA cohort,²⁵ with intra- and inter-assay variability of 2.7 and 8.6%, respectively. Urine creatinine was measured using the enzymatic method (COBAS Integra 400 Plus, Roche, Basel, Switzerland) where a standard range of 6–14 mmol/l was reported. Salivary cortisol and α-amylase concentrations were determined using commercial luminescence immunoassay kits (LIA) (IBL, Hamburg Germany) and inter-assay (< 5%) and intra-assay (< 4%) variability was reported.

For HPA sampling, fasting blood samples were obtained before 09:00 in both phases after the subjects had been awake for 90 minutes and in a semi-recumbent position.²⁵ Samples were handled according to standardised procedures and stored at –80°C until analysis. Serum cortisol and ACTH were analysed with an electrochemiluminescence immunoassay (e411, Roche, Basel, Switzerland). Normal ranges for ACTH are between 10 and 60 pg/ml.

For confounder biochemical analyses, serum high-density lipoprotein cholesterol (HDL-C), an ischaemic stroke risk marker,^{30–33} was analysed with an enzyme-rated method (Unicel DXC 800 – Beckman and Coulter, Germany). HDL-C ≥ 1.17 mmol/l is acceptable for normal HDL-C functioning, whereas ≤ 1.04 mmol/l reflects an increased risk for cardiovascular disease.³¹ Serum high-sensitivity C-reactive protein (CRP), serum gamma glutamyl transferase (γ-GT) and whole-blood EDTA glycated haemoglobin (HbA_{1c}) were analysed with turbidimetric inhibition immunoassays (Cobas Integra 400 Plus, Roche, Basel, Switzerland). Serum cotinine was analysed with a homogeneous immunoassay (Modular Roche automatised systems, Basel, Switzerland). Intra- and inter-assay coefficients for all analyses were less than 10%.

Statistical analysis

Statistica version 13.3 (TIBCO Software Inc, Palo Alto, USA, 2018) was used for data analyses. Three-way ANCOVAs independent of *a priori* covariates were computed to determine main effect interactions (race × gender × u-NE median/tertiles/quartiles/quintiles) for stroke^{4,18,26} and neuronal hyperactivity

risk markers.^{25–27} Retinal risk covariates were selected *a priori* and included age, waist circumference (WC), cotinine smoking

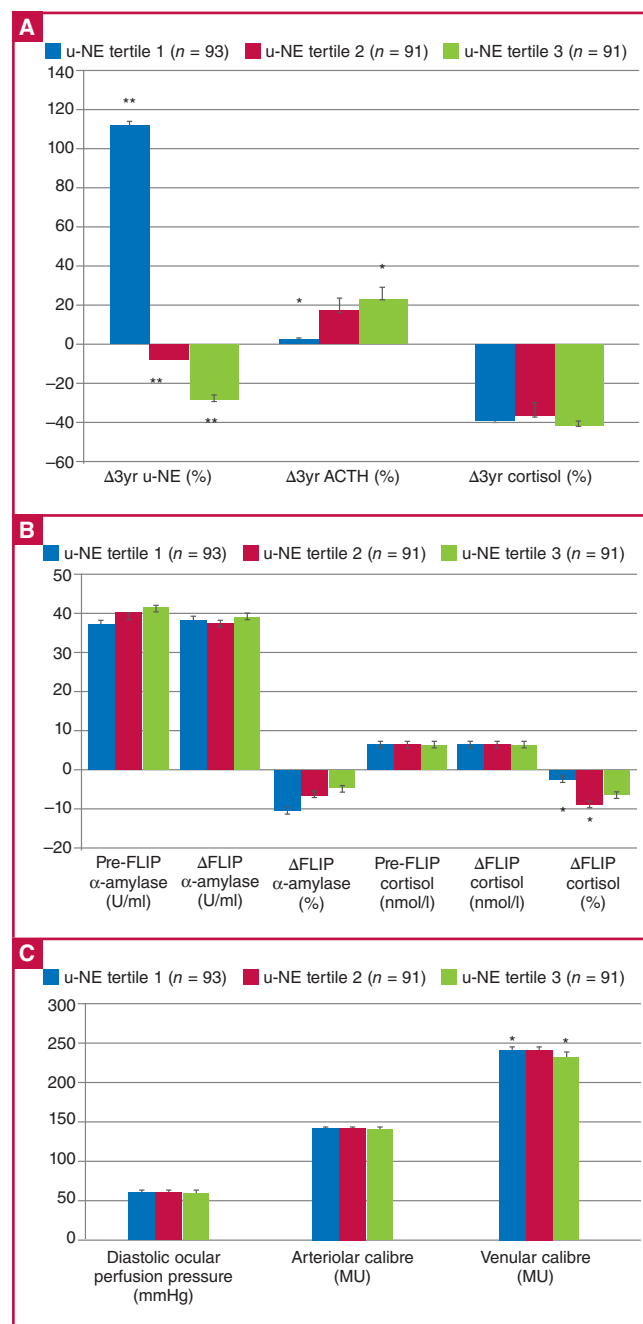


Fig. 2. Comparing stress hormones and retinal vessel risk markers. Data presented as median values across increasing u-NE tertiles (nmol/l: mmol/l) [u-NE tertile 1, median (min–max): 8.74 (1.05–14.77); u-NE tertile 2, median (min–max): 21.23 (15.05–28.62); u-NE tertile 3, median (min–max): 40.62 (28.69–113.63)]. A. Stress hormone three-year changes, with salivary stress hormone changes (B) prior to and upon flicker light-induced provocation (FLIP). C. Retinal vessel risk markers. Significance is shown using *p*-values of non-parametric Kruskal–Wallis tests. u-NE, norepinephrine:creatinine ratio; Δ3yr, three-year changes; ACTH, adrenocorticotrophic hormone. **p* ≤ 0.05; ***p* ≤ 0.001.

Table 2. Comparing unadjusted stress hormones, HDL-C and retinal vessel calibres across norepinephrine:creatinine (u-NE nmol/l: mmol/l) tertile groups

	Tertile 1	Tertile 2	Tertile 3	p-values		
	u-NE median (min-max): 8.74 (1.05–14.77) (n = 93)	u-NE median (min-max): 21.23 (15.05–28.62) (n = 9)	u-NE median (min-max): 40.62 (28.69–113.63) (n = 91)	u-NE tertiles 1 vs 3	u-NE tertiles 1 vs 2	u-NE tertiles 2 vs 3
3-yr stress hormones changes						
Δ3yr u-NE (%)	111.6 (4.1, 207.4)	-7.08 (-46.0, 53.4)	-27.8 (-54.0, 21.1)	0.01 (0.01)	0.01 (0.01)	0.01 (0.04)
Δ3yr ACTH (%)	2.5 (-30.1, 37.2)	16.1 (-20.6, 58.4)	26.2 (-15.8, 83.9)	0.02 (0.01)	0.25 (0.09)	0.91 (0.32)
Δ3yr cortisol (%)	-37.9 (-53.6, -17.7)	-34.0 (-49.8, -13.0)	-41.7 (-54.5, -19.3)	1.00 (0.59)	1.00 (0.32)	0.50 (0.19)
Δ3yr HDL-C (%)	-11.4 (-24.6, 0.81)	-13.3 (-27.6, 1.67)	-11.8 (-20.0, 4.88)	1.00 (0.63)	1.00 (0.79)	1.00 (0.48)
Saliva stress hormones prior to and upon FLIP						
Pre-FLIP α-amylase (U/ml)	36.8 (20.4, 73.9)	39.7 (17.5, 72.0)	41.4 (26.0, 62.2)	1.00 (0.89)	1.00 (0.87)	1.00 (0.83)
ΔFLIP α-amylase (U/ml)	38.3 (22.0, 64.3)	37.6 (19.3, 66.9)	38.8 (22.7, 68.4)	1.00 (0.89)	1.00 (0.89)	1.00 (0.76)
ΔFLIP α-amylase (%)	-9.79 (-35.3, 44.8)	-5.6 (-48.4, 64.5)	-4.30 (-36.7, 39.2)	1.00 (0.96)	1.00 (0.89)	1.00 (1.00)
Pre-FLIP cortisol (nmol/l)	5.5 (3.94, 8.05)	5.1 (3.55, 8.59)	5.1 (3.34, 7.04)	0.64 (0.21)	1.00 (0.50)	1.00 (0.59)
ΔFLIP cortisol (nmol/l)	5.3 (3.79, 8.11)	4.87 (3.38, 7.39)	4.7 (3.20, 6.65)	0.37 (0.12)	0.69 (0.24)	1.00 (0.74)
ΔFLIP cortisol (%)	-0.2 (-9.12, 7.28)	-8.2 (-14.9, 1.46)	-5.2 (-11.4, 3.48)	1.00 (0.33)	0.05 (0.02)	0.39 (0.10)
Structure: retinal arteries and veins						
Retinal artery (MU)	151.2 (143.1, 158.9)	152.1 (141.0, 160.0)	151.4 (141.7, 157.4)	1.00 (0.97)	1.00 (0.65)	1.00 (0.63)
Retinal vein (MU)	245.3 (231.9, 257.8)	243.1 (227.2, 261.0)	239.5 (226.7, 249.8)	0.17 (0.05)	1.00 (0.87)	0.29 (0.11)
Functionality: arteries						
Mean maximal arterial dilation, (% baseline)	3.7 (2.3, 5.2)	3.9 (1.9, 5.6)	3.6 (1.9, 5.3)	1.00 (0.85)	1.00 (0.70)	1.00 (0.57)
Time of maximal arterial constriction (from the start of flicker) (s)	44.5 (37.0, 56.5)	50.0 (43.0, 62.0)	48.0 (38.0, 68.0)	0.55 (0.24)	0.05 (0.01)	0.82 (0.35)
Functionality: veins						
Mean maximal venous dilation (% baseline)	3.9 (3.0, 5.0)	4.4 (3.1, 5.9)	3.8 (2.8, 4.7)	0.80 (0.37)	0.89 (0.30)	0.01 (0.03)
Post-FLIP vein recovery (% of baseline)	100.6 (100.3, 101.0)	100.5 (100.2, 100.9)	100.4 (100.1, 100.7)	0.10 (0.03)	1.00 (0.52)	0.44 (0.16)
Diastolic ocular perfusion pressure (mmHg)	69.0 (64.0, 75.0)	69.0 (59.0, 77.0)	69.0 (63.0, 75.0)	1.00 (0.07)	1.00 (0.46)	1.00 (0.61)

Data are presented as median values (inter-quartile ranges) whereas significance is shown using *p*-values of non-parametric Kruskal–Wallis tests followed by multiple comparison tests (uncorrected *p*-values of Mann–Whitney *U*-test).
 Δ3yr, three-year changes; FLIP, flicker light-induced provocation; ΔFLIP, changes during FLIP; ACTH, adrenocorticotrophic hormone; HDL-C, high-density lipoprotein cholesterol.

status, γ-GT, HbA_{1c}, HDL-C, hypertensive/diabetic retinopathy and DOPP.⁴¹⁸ An interaction term was fitted for retinal vein dilation [$F_{1,254} = 4.34$ ($p = 0.014$)] in u-NE tertiles, and not in median/quartiles or quintiles. Hence the cohort was stratified into baseline norepinephrine:creatinine (u-NE in nmol/l: mmol/l)

ratio tertile groups supporting assessment for SAM activity/ adrenergic drive and potential monoamine depletion.³³⁻³⁶ The respective median (min-max) u-NE concentrations were: u-NE

Table 3. Forward stepwise linear regression analyses depicting associations between retinal vessel calibres, stress hormones and risk markers in norepinephrine:creatinine (u-NE nmol/l: mmol/l) tertile 1

	u-NE tertile 1 median (min-max): 8.74 (1.05–4.77) (n = 92)	
	Arteries (MU)	Veins (MU)
Δ3yr stress hormones (%)		
Adjusted R ²	0.32	0.38
	β (95% CI)	β (95% CI)
Δ3yr u-NE (%)	-	-
Δ3yr cortisol (%)	-	-
Baseline HDL-C (mmol/l)	-0.20 (-0.38, -0.02), <i>p</i> = 0.040	-
Stress hormone levels prior to FLIP		
Adjusted R ²	0.26	0.33
	β (95% CI)	β (95% CI)
Saliva α-amylase (U/ml)	-0.28 (-0.50, -0.06), <i>p</i> = 0.010	-
Saliva cortisol (nmol/l)	-	-0.33 (-0.53, -0.13), <i>p</i> = 0.002
Diastolic ocular perfusion pressure (mmHg)	-0.24 (-0.46, -0.02), <i>p</i> = 0.024	-

Δ3yr; three-year stress hormone changes (%); Prior to FLIP, saliva stress hormone levels prior to FLIP. Δ, changes; FLIP, flicker light-induced provocation; HDL-C, high-density lipoprotein cholesterol.
 Additional covariates included age, waist circumference, cotinine smoking status, log-normalised gamma-glutamyl transferase, glycated haemoglobin, hypertensive/diabetic retinopathy, diastolic ocular perfusion pressure and the respective retinal artery/vein diameter.

Table 4. Ocular media and fundus assessment at three-year follow up across norepinephrine:creatinine (u-NE nmol/l: mmol/l) tertiles

	Count, prevalence (%)		
	u-NE tertile 1 (n = 93)	u-NE tertile 2 (n = 91)	u-NE tertile 3 (n = 91)
Referred to ophthalmologist	6 (6.7)	7 (7.8)	11 (12.5)
Hypertensive/diabetic retinopathy	41 (44.6)	47 (51.7)	54 (59.3)*
Retinopathy included, n (%)			
Intra-ocular pressure < 11 mmHg	12 (13.3)	8 (8.9)	9 (10.2)
Optic head (cup:disc ratio > 0.50)	17 (18.9)	12 (13.5)	21 (24.1)
Optic nerve head damage [†]	8 (8.9)	9 (10.2)	6 (6.7)
Acute anterior glaucoma risk	1 (1.1)	6 (6.7)	8 (9.1)
Retinal atrophy	2 (2.2)	0 (0.00)	0 (0.0)
Drusen	0 (0.0)	1 (1.1)	0 (0.0)
Ciliary blood vessels	1 (1.1)	0 (0.0)	0 (0.0)
Exudates	0 (0.0)	1 (1.1)	0 (0.0)
Haemorrhaging	4 (4.4)	1 (1.1)	1 (1.1)
Arteriovenous nicking	31 (33.7)	38 (41.8)	39 (42.9)
Neovascularisation	1 (1.1)	0 (0.0)	0 (0.0)
Cotton wool ischaemia	0 (0.0)	0 (0.0)	0 (0.0)
Focal narrowing	1 (1.1)	2 (2.2)	1 (1.1)
Macula scarring	1 (1.1)	0 (0.0)	0 (0.0)

Chi-squared statistics were used to determine prevalence in u-NE tertile 1 vs u-NE tertile 3 [u-NE tertile 1, median (min-max): 8.74 (1.05–4.77); u-NE tertile 2, median (min-max): 21.23 (15.05–28.62); u-NE tertile 3, median (min-max): 40.62 (28.69–113.63)].
[†]Optic nerve head damage, cup-to-disc ratio ≥ 0.3 plus intra-ocular pressure ≥ 21 mmHg. **p* ≤ 0.05.

Table 5. Forward stepwise regression analyses depicting associations between retinal vessel and stress hormone responses prior to and post flicker light-induced provocation (FLIP) in norepinephrine:creatinine (u-NE nmol/l:mmol/l) tertile 1

	u-NE tertile 1 median (min-max): 8.74 (1.05–14.77) (n = 93)			
	Artery max dilation (%)	Artery time max constriction (s)	Vein max dilation (%)	Vein post-FLIP recovery to baseline (%)
$\Delta 3\text{yr}$ stress hormones (%)				
Adjusted R^2	0.16	0.15	0.20	< 0.10
β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
u-NE (%)	-0.20 (-0.4, -0.0), $p = 0.055$	-	-	-
Serum cortisol (%)	-	-	-	-
Stress hormone levels prior to FLIP				
Adjusted R^2	< 0.10	0.14	< 0.10	< 0.10
β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Saliva α -amylase (U/ml)	-	-0.25 (-0.5, 0.0), $p = 0.029$	-	-
Δ FLIP stress hormones (%)				
Adjusted R^2	< 0.10	0.15	0.22	0.15
β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Saliva α -amylase (%)	-	NS	-	-
Saliva cortisol (%)	-	NS	-0.34 (-0.6, -0.1), $p = 0.003$	-0.25, (-0.5, 0.0), $p = 0.028$
Baseline HDL-C (mmol/l)	-	-	0.26 (0.0, 0.1), $p = 0.022$	-0.33 (-0.6, -0.1), $p = 0.004$

$\Delta 3\text{yr}$; three-year stress hormone changes (%); Prior to FLIP, saliva stress hormone levels prior to flicker light-induced provocation/FLIP; Δ FLIP, stress hormone changes (%) obtained directly after FLIP. Δ , changes; NS, non-significant, HDL-C; high-density lipoprotein cholesterol. Additional covariates included age, waist circumference, cotinine smoking status, log-normalised gamma-glutamyl transferase and glycated haemoglobin; hypertensive/diabetic retinopathy and diastolic ocular perfusion pressure.

Table 6. Logistic regression analysis to predict the probability of chronic stress-related stroke risk in a cohort with low urinary norepinephrine:creatinine (u-NE nmol/l:mmol/l) (n = 90)

	Chronic stress-related stroke risk			
	Odds ratio	5th percentile	95th percentile	p-value
Nagelkerke R^2 0.62				
DOPP (mmHg)	1.07	0.99	1.17	0.104
FLIP HRV (ms)	1.52	0.42	1.05	0.078
FLIP cortisol (%)	1.08	0.74	1.58	0.705
FLIP delayed venous recovery (% of baseline)	4.82	1.18	19.59	0.028

Covariates included high-density lipoprotein cholesterol and hypertensive/diabetic retinopathy. DOPP, diastolic ocular perfusion pressure; FLIP HRV, time-domain heart-rate variability standard deviation of the normal-to-normal (NN) intervals between adjacent QRS complexes, which equal the square-root of variance.

tertile 1 median (min-max): 8.74 nmol/l:mmol/l (1.05–14.77); u-NE tertile 2 median (min-max): 21.23 nmol/l:mmol/l (15.05–28.62); u-NE tertile 3 median (min-max): 40.62 nmol/l:mmol/l (28.69–113.63). Normality was tested and skewed data were log₁₀ normalised.

The clinical characteristic proportions of the cohort were determined at baseline using chi-squared (χ^2) statistics. Stress risk-marker changes were computed with dependent sample *t*-tests. Kruskal–Wallis tests determined significance, followed by multiple comparisons tests and non-parametric Mann–Whitney *U*-tests when comparing retinal and stress hormone median values.

Multiple linear regression analyses determined associations between the retinal vasculature and the stress hormones in each of the u-NE tertiles. Retinal vessel responses upon provocation (FLIP) included four dependent variable models: artery maximal dilation (%), artery time to constrict (s), vein maximal dilation (%) and vein post-FLIP recovery to baseline value (%). The retinal vessel calibre models included two dependent variable

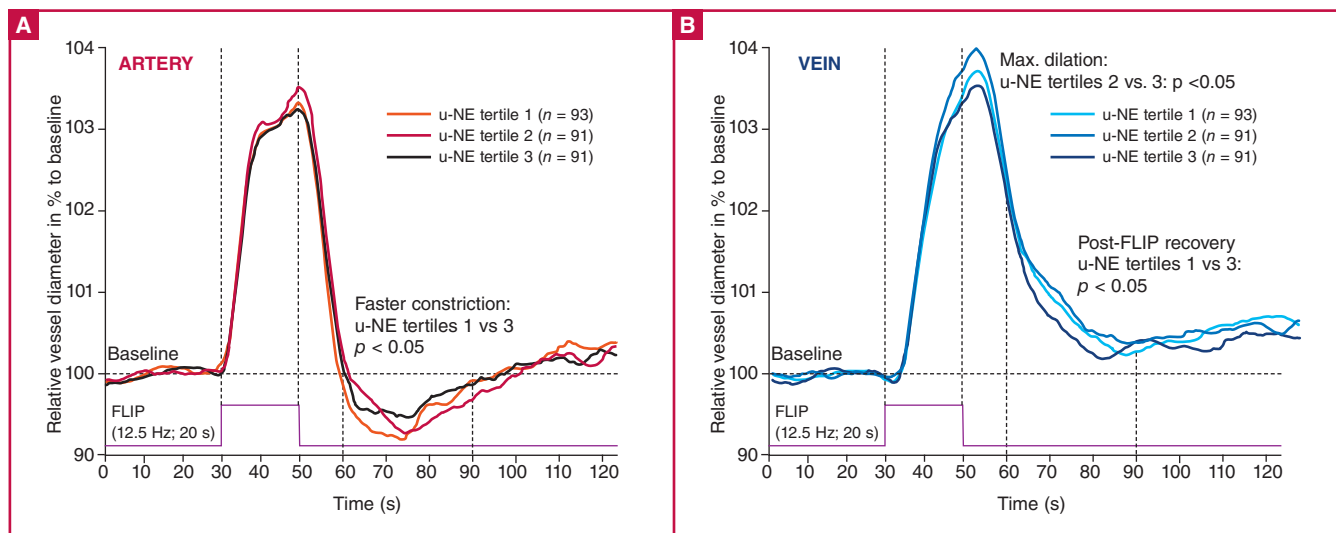


Fig. 3. Comparing median retinal artery (A) and vein (B) responses during monochromatic flicker light-induced provocation (FLIP) in increasing norepinephrine:creatinine (u-NE nmol/l:mmol/l) tertiles. Exact *p*-values were determined with Mann–Whitney *U*-tests. Grey vertical stripes indicate the time interval 10–40 seconds after flicker cessation where arterial constriction and an emphasised decrease of venous diameter are expected. Delayed vein recovery responses (% of baseline) were determined by calculating the average vessel diameter between 50 and 80 seconds after the end of flicker. u-NE tertile 1, median (min-max): 8.74 (1.05–14.77); u-NE tertile 2, median (min-max): 21.23 (15.05–28.62); and u-NE tertile 3, median (min-max): 40.62 (28.69–113.63).

models: retinal arteries and veins. Independent variables for these six models included *a priori* covariates, the respective retinal artery/vein diameter and stress hormone responsiveness (1) difference over three years ($\Delta 3\text{yrs}$) (%), (2) prior to FLIP, and (3) upon provocation (Δ FLIP%). Multiple linear regression analyses were repeated by controlling for HRV, physical activity and the use of cortisone derivatives, α - and/or β -blockers at follow up.

We recently developed a method of determining risk for chronic stress and stroke (filed 31 July 2020, international patent application no. PCT/IB2020/05726). We applied this score to determine whether retinal vascular responses would predict chronic stress and stroke risk. Logistic regression analyses were computed and included the covariates: stress hormone

changes over three years or upon provocation, HRV, diastolic ocular perfusion pressure, HDL-C and hypertensive/diabetic retinopathy. The statistical significance level was set at $p \leq 0.05$ (two-tailed). The F to enter in regression models was fixed at 2.5.

Results

Tertile characteristics (Table 1) showed an increasing trend across u-NE tertiles for central obesity and decreasing trends in cortisol and HDL, particularly in u-NE tertile 1. Again in u-NE tertile 1, consistent inflammation (CRP) and raised BP were observed, whereas a decrease occurred in u-NE tertile 3.

Stress hormones: Fig. 2A and Table 2 (median \pm 95% CI) show u-NE increases in u-NE tertile 1 (111.6%) but decreases in u-NE tertiles 2 and 3 over three years ($p \leq 0.01$). ACTH levels did not change in u-NE tertiles 1 and 2, however the increase in u-NE tertile 3 was higher compared to tertile 1 ($p \leq 0.001$). In u-NE tertile 1 (Fig. 2B), saliva post-FLIP cortisol (%) was lower compared to u-NE tertile 2 ($p \leq 0.05$). Vein widening (Fig. 2C) was apparent in u-NE tertile 1 (245.3 MU) compared to u-NE tertile 3 (239.5 MU) ($p \leq 0.05$). In Table 2, medians were compared and a five-second faster arterial constriction (Fig. 3A) was evident in u-NE tertile 1 compared to u-NE tertile 2 ($p \leq 0.05$). In Fig. 3B, veins dilated significantly more in u-NE tertile 2 when compared to u-NE tertile 3 ($p \leq 0.05$). The venous post-FLIP recovery response was delayed ($p \leq 0.05$) in u-NE tertile 1 compared to u-NE tertile 3. In Table 4, hypertensive/diabetic retinopathy was higher in u-NE tertile 3 compared to tertile 1.

Stress hormones and retinal vasculature associations: multiple stepwise linear regression associations between retinal vessel calibres (Table 3) and retinal FLIP responses (Table 5), and stress hormones of u-NE tertile 1 are presented. Reduced arterial dilation, faster constriction, narrowing and hypo-perfusion were associated with increased SAM activity. Delayed venous dilation, recovery and widening were associated with cortisol hypo-secretion and low HDL-C ($p \leq 0.05$).

In u-NE tertile 1 (Table 6), delayed vein recovery responses predicted stress and stroke risk, having large clinical significance [odds ratio 4.8 (1.2–19.6); $p = 0.03$]. Associations between the retinal vasculature and cortisol secretion in u-NE tertiles 2 and 3 showed effective cortisol functioning but no relationship existed with norepinephrine (Table 7). Controlling for HRV, physical activity and the use of cortisone derivatives, α - and/or β -blockers at follow up did not change the outcome of our findings.

Discussion

We aimed to (1) assess the relationships between the retinal vasculature, SAM and HPA activity over three years and upon provocation, and (2) determine chronic stress and stroke risk. Findings showed that in the presence of low norepinephrine, a reflex increase in SAM activity occurred, enhancing arterial vasoconstriction and hypo-perfusion. Concomitant HPA dysregulation attenuated retinal vein vasoactivity and tone, reflecting delayed vein recovery responses and non-adaptation to stress. These constrained vein recovery responses demonstrated increased chronic stress and stroke risk, having large clinical significance. The main findings are presented below (Fig. 4).

Table 7. Forward stepwise regression analyses depicting associations between retinal vessel and stress hormone responses prior to and post flicker light-induced provocation (FLIP) in norepinephrine:creatinine (u-NE nmol/l:mmol/l) tertiles 2 and 3

<i>u-NE tertile 2 median (min-max): 21.23 (15.05–28.62) (n = 87)</i>				
	Artery max dilation (%)	Artery time max constriction (s)	Vein max dilation (%)	Vein post-FLIP recovery (% of baseline)
$\Delta 3\text{yr}$ stress hormones (%)				
Adjusted R^2	0.25	< 0.10	0.20	< 0.10
	β (95% CI)		β (95% CI)	β (95% CI)
u-NE (%)	–	–	–	–
Serum cortisol (%)	0.21 (0.03, 0.39)*	–	–	–
Stress hormone levels prior to FLIP				
Adjusted R^2	0.25	< 0.10	< 0.10	< 0.10
	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Saliva cortisol (nmol/l)	–0.26 (–0.46, –0.06)*	–	–	–
Δ FLIP stress hormones (%)				
Adjusted R^2	0.29	< 0.10	0.25	0.15
	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Saliva α -amylase (%)	–	–	–	–
Saliva cortisol (%)	–	–	–	–0.36 (–0.60, –0.13)*
<i>u-NE tertile 3 median (min-max): 40.62 (28.69–113.63) (n = 89)</i>				
	Artery max dilation (%)	Artery time max constriction (s)	Vein max dilation (%)	Vein post-FLIP recovery (% of baseline)
$\Delta 3\text{yr}$ stress hormones (%)				
Adjusted R^2	0.22	0.12	0.20	< 0.10
	β (95% CI)		β (95% CI)	β (95% CI)
u-NE (%)	–	–	–	–
Serum cortisol (%)	–	–0.22 (–0.42, –0.02)*	–	–
Stress hormone levels prior to FLIP				
Adjusted R^2	< 0.10	0.11	< 0.10	< 0.10
	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Saliva cortisol (nmol/l)	–	– NS	–	–
Δ FLIP stress hormones (%)				
Adjusted R^2	0.15	0.17	0.15	< 0.10
	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)
Saliva α -amylase (%)	–	–	–	–
Saliva cortisol (%)	–	–	–	–

$\Delta 3\text{yr}$: three-year stress hormone changes (%); Prior to FLIP, saliva stress hormone levels prior to FLIP; Δ FLIP, stress hormone changes (%) obtained directly after FLIP. Δ , changes.

Additional covariates included age and log-normalised waist circumference, cotinine, gamma-glutamyl transferase and glycated haemoglobin; hypertensive/diabetic retinopathy, diastolic ocular perfusion pressure and the respective retinal arterial/vein diameter.

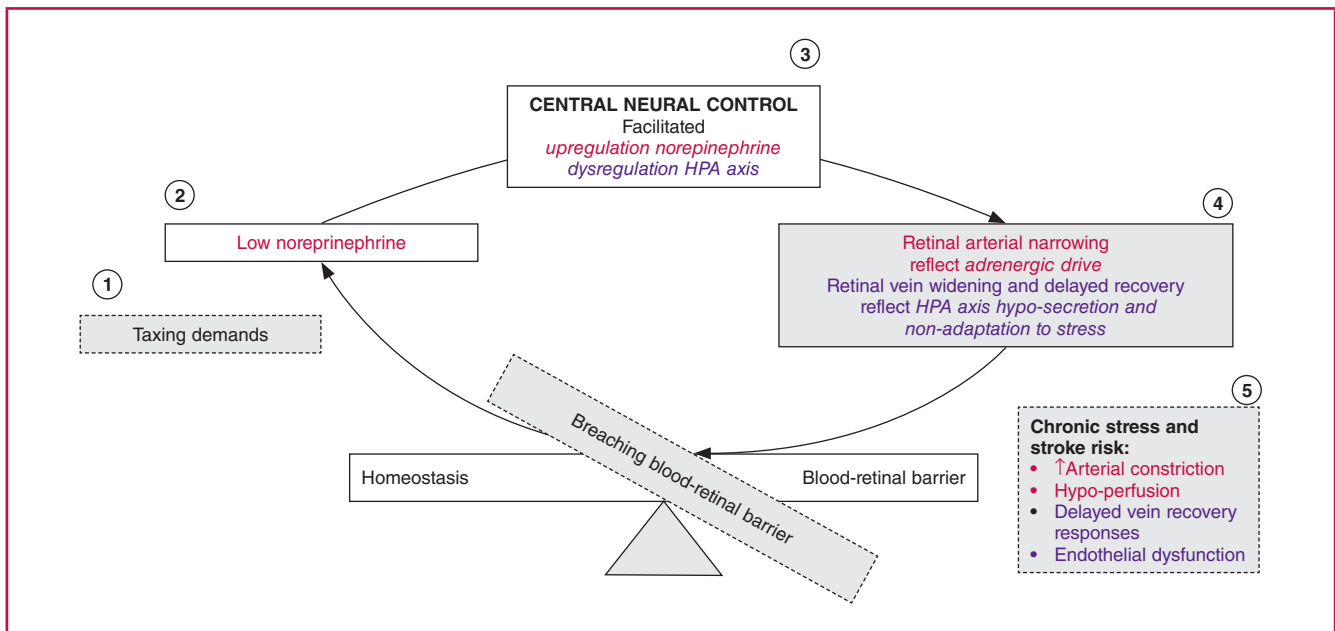


Fig. 4. Graphical representation of the main findings indicating the relationship between chronic stress-related stroke risk and retinal vein recovery responses.

Norepinephrine and the retinal vasculature

In 2008, Krishnan and Nestler³⁶ discussed the monoamine hypothesis of depression, which posits that depression is caused by decreased monoamine function in the brain. Findings in the low-u-NE group (tertile 1) support this low monoamine premise, as initial low norepinephrine or monoamine concentrations facilitated central neural control. An upregulation of norepinephrine occurred over three years, resulting in neuronal hyperactivity or adrenergic drive. In support, Ferrier *et al.*⁶ suggested that the presence of chronic stress increases human sympathetic firing, which is dependent on norepinephrine release within the brain and the activation of central neural control mechanisms to maintain homeostasis.^{34,36} Catecholamine surges following systemic insults, such as stress, is further directly involved in the regulation of cytokine expression and exemplifies the consistently high CRP level of 5 mg/l in the u-NE tertile 1, a worsening clinical condition.³³⁻³⁶

The noradrenergic cell groups A6 (locus coeruleus) project axons to the hypothalamic paraventricular nucleus (PVN) to activate the SAM and promote norepinephrine release during acute and chronic stress.^{11,36} However, the specific neurovascular coupling mechanism in the human retina when neuronal hyperactivity and chronic stress are apparent are not quite so clear. Retinal neurons such as the amacrine and horizontal cells synthesise catecholamines,¹⁰ as well as high-affinity α_{1a} -AR and α_{2a} -AR, which are expressed in Müller and ganglion cells, and the inner plexiforme, inner nuclear and photoreceptor layers of the retina.^{10,22,37-39} Upon activation of α_{2a} -AR, norepinephrine release is inhibited to protect ganglion cells against disturbed ocular perfusion pressure⁴⁰ and arterial occlusion by reducing intracellular cyclic adenosine monophosphate (cAMP) production.^{3,5} Müller cells and other astrocytes have intimate contact with both synapses and blood vessels via α_{2a} -AR activity, which enables regulation of blood flow.^{41,42}

A cycle of events may occur where chronic stress as the initial trigger reflects low norepinephrine and potential

monoamine depletion (in u-NE tertile 1), and where central homeostatic reflexes are activated to facilitate upregulation of an endogenous catecholamine, norepinephrine. This happens via activation of the hypothalamic PVN with sensitisation of α_{1a} -AR (vasoconstriction) and desensitisation of vasodilatory α_{2a} -AR in the retina. This may indicate that chronic stress induces central neural control mechanisms, potentially overriding autoregulation in the retina. Furthermore, norepinephrine may also bind with dopamine 2 receptors (D_2R) as potential signal transducers for norepinephrine in the outer and inner retinal nuclear layers.⁴³ Retinal dopamine is synthesised and released from dopaminergic amacrine cells⁴³ and binds to high-affinity D_2R to activate norepinephrine release.^{43,44} In support of this notion, Jäkel and Dimou⁴⁵ reported that hetero-receptor cellular communication occurs between the different glial cell types under pathological conditions.

Chronic stress may be such a pathological condition,⁴ as central neural control with upregulation of norepinephrine in u-NE tertile 1 may have decreased α_{2a} -affinity/specificity. This may allow D_2R receptors to relay higher norepinephrine vasoconstrictive signalling, thereby inducing reduced arterial dilation, faster constriction, narrowing and hypo-perfusion in u-NE tertile 1. Another study demonstrated desensitisation of α_{1a} -AR upon provocation to protect the BRB,¹⁰ potentially explaining the lack of saliva α -amylase responses in our low-u-NE cohort. This lack of variation in saliva α -amylase upon provocation concurs with another study's findings where chronic stress or burn-out was associated with attenuated α -amylase responses.⁴⁶ An adrenergic drive marker, depressed HRV, was also suggested as an objective stress marker.³⁵ However, we could not confirm HRV as a risk marker for either chronic cardiomyocyte injury²⁵⁻²⁷ or chronic stress and stroke risk in the current cohort.

The observed adrenergic drive or neuronal hyperactivity in u-NE tertile 1 increased arterial vascular resistance and tone, and may impair myogenic control. Considering the chronic pre-diabetic status and adrenergic drive in u-NE tertile 1

individuals, a profile of endothelial dysfunction⁴⁷ emerges, which could enable the α_{1b} -adrenoceptor subtype to mediate adrenergic vasoconstriction in retinal arteries.⁴⁸ The overall reflex increases in u-NE, and pre-FLIP α -amylase levels support the notion of adrenergic-induced vasoconstriction. These changes have presumably been mediated via the following catecholamine receptors: α_{1a} -AR, α_{1b} -AR, desensitised α_{2a} -AR and D_2R .^{10,22,37,39,43,44} Such a profile compromises the integrity of the BRB³³ and increases susceptibility for ischaemic stroke risk.^{15,16,34}

Cortisol and the retinal vasculature

Compared with SAM responses, the HPA has a particularly high threshold for activation^{12,49} and facilitates the ability to cope with, adapt to, and recover from stress.² A homeostatic negative feedback response occurred in u-NE tertile 3, where increases in ACTH and decreases in cortisol levels were observed, and which were not related to retinal vascular dysregulation. In u-NE tertile 1, however, no increases in ACTH level occurred despite decreases in cortisol level. This potentially reflects a positive feedback response of HPA hypo-activity, which is indicative of chronic uncontrollable stress.^{12,49} In rodent models a low norepinephrine level¹⁰ in the central amygdala and hypothalamus (PVN) inhibited HPA responses to neural stress stimuli such as photic stimulation.⁵⁰ This might explain attenuation of the negative feedback exerted by glucocorticoids in u-NE tertile 1, probably by reducing hippocampal glucocorticoid receptors and facilitating consistently raised ACTH levels.

HPA regulation, particularly by cortisol, protects neurons by suppressing inflammation and inhibiting microglial TNF- α release and GCR signalling to permit the formation of proliferating Müller glia-derived progenitor cells.^{12,51} Cortisol hypo-secretion in adrenergic-driven u-NE tertile 1 might, therefore, reduce immune function suppression and explain the observed low-grade inflammation and retinal vein widening. Retinal veins consist of a single layer of endothelial cells and few smooth muscle cells.⁵² Retinal vein vascular tone and diameter changes in u-NE tertile 1 may therefore affect vein drainage resistance and upstream pressure in retinal capillaries. Indeed, HPA dysregulation reflected lower vein vasoactivity and tone, which delayed recovery responses upon provocation and implies non-adaptation to stress.

In support, the observed prolonged stress responses in hippocampal (ventral subiculum) lesions of rats were accompanied by enhanced depletion of corticotrophic hormone immunoreactivity over time² and, in humans, were related to neurodegenerative disease risk (depression and the late onset of Alzheimer's disease).⁵³ In a previous study, retinal vein widening reflected self-reported chronic depressive symptoms and a compromised NO-synthase system in a black cohort.¹⁸ Presently, HPA dysregulation in u-NE tertile 1, independent of race and gender, might decrease nitric oxide (NO) bioavailability by inhibiting NO-synthase directly⁵⁴ and explain compromised vein dynamics in the retina.⁵⁴ In the current low u-NE group, retinal vein responses were not related to self-reported chronic depression, but only to stress hormone dysregulation. This discrepancy and the lack of association between cardiovascular disease risk markers and self-reported chronic depression concurs with previous observations,^{4,27} as well as with the most recent findings by Levis *et al.*⁵⁵ Applying a validated chronic

stress and stroke risk marker may prove to be superior to self-reported chronic depression.

Stress and ischaemic stroke susceptibility

Small vessels in the brain react to hydrostatic pressure and will regulate vascular tone to maintain a constant blood flow, or autoregulation.³⁴ Central control with dysregulated HPA and downregulated HDL-C enhanced endothelial dysfunction in u-NE tertile 1, increasing susceptibility for ischaemic stroke.¹⁵ The greater FLIP venous dilation in u-NE tertile 1, slower recovery to baseline and widening have now been associated with both cortisol hypo-secretion and low HDL-C levels. This may impede constant blood flow and autoregulation, and facilitate ischaemic stroke susceptibility.¹⁵ Central control may indeed override autoregulation when chronic stress is apparent.

Neuronal hyperactivity may drive and dominate these responses as low HDL-C level in turn influences cerebrovascular function and breaching of the BRB.^{10,13-15} Intra-retinal lipid transport depends on HDL-C, the major apolipoprotein constituent of apolipoprotein E (ApoE).⁵⁶⁻⁵⁸ The 22% decrease (1.1–0.9 mmol/l) observed in u-NE tertile 1 may be indicative of a high risk (< 1.04) for depression,⁵⁶ retinal pathology,⁵⁸ cognitive decline⁵⁹ and ischaemic stroke.⁶⁰ Low HDL-C levels reflected chronic stress⁵¹ and clinical depression,⁶¹ endorsing the prevalence of chronic stress in u-NE tertile 1. It is important to note that certain depression treatments may downregulate norepinephrine, such as tricyclic antidepressants (e.g. serotonin re-uptake inhibitors). Subsequent upregulation of norepinephrine can occur, which will disturb neurovascular coupling⁶² and potentiate stroke risk.

Neural mechanism for chronic stress and stroke risk

In response to low norepinephrine levels, a reflex increase in sympathetic activity/adrenergic drive occurred as a compensatory mechanism to low monoamine levels. Higher adrenergic drive may potentiate catecholamine receptor sensitisation (potentially α_{1a} -AR, α_{1b} -AR, D_2R) and/or chronic α_{2a} -AR desensitisation.^{10,22,37-39,43,44} Indeed, higher adrenergic drive increased vasoconstriction and hypo-perfusion or ischaemia in retinal arteries. Consistent high blood pressure and vasoconstrictive signalling may exert deleterious effects on the retinal ganglion cells.⁴⁰ Concomitant HPA dysregulation, resembling uncontrollable stress, was related to delayed venous dilation, recovery and widening. Most prominently, the delayed venous recovery upon provocation may indicate a prolonged retrograde propagation of the vascular response, reflecting lower vein vaso-activity and tone. Consistent low-grade inflammation will further increase the risk for endothelial dysfunction, a breach in BRB,³⁴ as well as ischaemic stroke.⁴ Delayed vein recovery responses upon provocation suggest non-adaptation to stress, which constrained recovery and autoregulation. Indeed, delayed retinal venous recovery predicted chronic stress and stroke risk (OR 4.8), having large clinical significance.

Limitations

Our study is limited as the sample size was relatively small and it should be repeated in larger longitudinal studies. However, within a well-controlled setting, we were able to apply (1) an

innovative approach by determining HRV and salivary stress hormone levels upon provocation to substantiate SAM and HPA activity, and (2) a clinical stress and stroke risk score. Our findings contribute to the sparse information on neural mechanisms and chronic stress-induced stroke risk in the human brain–retina axis.

Conclusion

In response to low norepinephrine levels, a reflex increase in sympathetic activity occurred, resulting in increased norepinephrine levels and hypo-perfusion, potentiating risk for retinal ganglion cell health. Concomitant HPA dysregulation changed retinal vein dynamics as delayed recovery responses reflected non-adaptation to stress. Indeed, constrained or delayed venous recovery responses predicted chronic stress and stroke risk.

The ethics on publishing scientific articles were followed. We gratefully acknowledge the voluntary collaboration of the participants. The SABPA study would not have been possible without the valuable contributions from co-investigators and technical staff.

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WV is chief executive officer of the Imedos Systems GmbH (Jena, Germany) and a freelance researcher. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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