Neural control, cardiac stress and retinal vascular dynamics:

The SABPA study

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"For by Him were all things created, that are in heaven, and that are in earth, visible and invisible, whether they be thrones, or dominions, or principalities, or powers: all things were created by Him, and for Him: And He is before all things, and by Him all things consist"

Colossians 1:16-17 (KJV)

"... want in Hom is alle dinge geskape wat in die hemele en op die aarde is, wat sienlik en onsienlik is, trone sowel as heerskappye en owerhede en magte – alle dinge is deur Hom en tot Hom geskape. En Hy is voor alle dinge, en in Hom hou alle dinge stand."

Kolossense 1:16-17 (Afrikaanse 1933/1953 Vertaling)

This thesis is dedicated to

Professors Nico and Leoné Malan

Pioneers that have set the path on which we may embrace the future

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Titel

Neurale beheer, kardiale stres en retinaal vaskulêre dinamika: Die SABPA-studie

Motivering

Strukturele en funksionele ooreenkomste bestaan tussen die retinale, serebrale en koronêre vaskulatuur. Die retinale en serebrale vaskulatuur vertoon besonderse anatomiese, funksionele en outoregulatoriese eendershede – sodoende word konstante bloeddruk (BP) gehandhaaf, ten spyte van sistemiese veranderinge in BP. Die laasgenoemde verseker genoegsame perfusie en beskerming van die bloed-okulêre en bloed-brein skanse. Die vermoë om hierdie outoregulatoriese kapasiteite te onderhou verswak egter met toenames in BP. Simpatiese senuweestelsel (SSS) hiperaktiwiteit in respons op beide chroniese en akute stressors, mag ’n risikofaktor vir hipertensie, ischemiese beroerte en ander kardiovaskulêre siektetoestande (KVS) wees; spesifiek in Afrikane. Hierdie toestande affekteer primêr die mikrovaskulatuur. Die retina is die enigste posisie waar die struktuur en funksie van die mikrovaskulatuur nie-ingrypend waargeneem kan word. Aansienlike omstredenheid bestaan egter rakende die legitimiteit van eksterne faktore wat moontlik die miogene, neurogene en neurovaskulêre koppelingsmeganismes moontlik kan beïnvloed of bepaal – en hierdeur dus die vermoë van die retinale vaskulatuur om selfregulerend op te tree, affekteer. Studies wat SSS-hiperaktiwiteit, merkers van kardiale stres en die assosiasie van neurotrofiene met die retinale vaskulatuur ondersoek, is skaars of ontbreek geheel-en-al. ’n Sogenaamde brein-retina-hartskakel kan as ’n moontlike meganistiese weg, binne die konteks van KVS, miokardiale ischemie en beroerte, bestudeer word.

Doelstellings

Die primêre doel van hierdie studie was om die invloed van SSS-hiperaktiwiteit, kardiale stres en neurotrofiese faktore op die struktuur en funksie van die retinale vaskulatuur binne ’n Suid-Afrikaanse bi-etniese kohort te evalueer. Hierdie innoverende benadering regverdig ’n stap-vir-stap-evaluering en verkenning van die brein-retina-hartskakel om sodoende ’n meer holistiese meganisme te identifiseer en dit hoofsaaklik oor te dra op beroertevatbaarheid-risiko. Die studie, *Simpatiese aktiwiteit en Ambulatoriese Bloeddruk in Afrikane* (SABPA), was ideaal om hierdie doelwitte onder die loep te neem, aangesien dit die enigste studie in Sub-Sahara Afrika is wat ontwerp is om die brein-hartskakel te ondersoek, via retinale vaskulêre struktuur en funksie, kardialestres-merkers, geaffilieerde neurotrofiese faktore, kardiovaskulêre en beroerte-risiko, in ’n bi-etniese groep.

Metodologie

Hierdie studie is geaffilieer met die SABPA-studie. Deelnemers was Afrikane en Kaukasiese onderwysers (N=409) wat gewerf is uit die Noordwes-Provinsie (Dr Kenneth Kaunda Onderwys Distrik), Suid-Afrika, almal met soortgelyke sosio-ekonomiese, onderrig- en gesondheidsorg-status. Alle deelnemers in fase 1 (2007-2008) is genooi om aan fase 2 (2011-2012) deel te neem. Van die aanvanklike 409 deelnemers het 359 teruggerapporteer vir fase 2 van die studie. Slegs deelnemers wat aan fase 2 deelgeneem het, is ingesluit, aangesien geen retinalevat-analises tydens fase 1 uitgevoer is nie. Van hierdie deelnemers is n=41 swak/ onbruikbare retinalevat-opnames en n=1 epilepsie van alle analises uitgesluit. Bykomende uitsluitings is gemaak in die onderskeie manuskripte ooreenkomstig die vasgestelde hipotese van elk. Groepe is ingedeel volgens etnisiteit soos bewys deur statisties betekenisvolle interaksies tussen die hoofveranderlikes. Die Dinamiese-Retinalevat-Analiseerder (DVA) is gebruik om die retinalevat-struktuur (sentrale retinale arteriolêre ekwivalent (CRAE), sentrale retinale venule ekwivalent (CRVE) en arteriolêre:venule ratio) en funksie (arteriolêre en venule maksimale dilatasie, arteriolêre maksimale konstriksie, tyd om te dilateer, en om te konstrikteer en die area-onder-die-kromme- (AUC) analises tydens flikkerlig-geïnduseerde provokasie (FLIP) te assesseer. Sistemies kardiale stresmerkers, gemeet in serum, het kardiale troponien T (cTnT) en amino-terminaal pro-B-tipe natriuretiese peptide (NT-proBNP) ingesluit. Harttempovariasie-parameters (HRV) het goue-standaard tyd- en frekwensiedomein-parameters, gemeet oor 24 uur en tydens FLIP, ingesluit. Brein-afgeleide neurotrofiese faktor (BDNF) is gemeet in serum monsters. Retinale venuleverwyding, arterio-venule "nicking", cTnT waardes bo 4.2ng/mL, NT-proBNP vlakke onder die ouderdoms- en geslagspesifieke verwysingsgrense en BDNF-vlakke onder 1.51ng/mL is toegepas ter berekening van die Universiteit van Kalifornië (UCLA) Beroerte risiko-telling, wat verhoogde 10-jaar beroertewaarskynlikheid assesseer. *A priori* koveranderlikes het oor die algemeen ouderdom, liggaamsoppervlak-area (BSA), serum kotinien, gamma-glutamiel transferase, hemoglobien A1c, totale cholesterol/ HDL-cholesterolratio, tumor-nekrotiese faktor-alfa, hipertensiewe/diabetiese retinopatie en 24-uur polsdruk. Bykomende aanpassings is vir CRVE in CRAE-modelle en omgekeerd gemaak. Statistiese berekeninge het T-toetse, analise van kovariate en Chi-kwadraattoetse ingesluit. Meervoudige liniêreregressie-analises het die onafhanklike verhouding tussen die hoofveranderlikes bepaal, terwyl waarskynlikheidsverhoudings (OR) die beroerterisiko-waarskynlikheid, soos gebaseer op die UCLA-beroerterisiko-telling, onafhanklik van *a priori* veranderlikes, bepaal het. "*Receiver operating characteristics*" (ROC) is toegepas om die optimale BDNF-afsnypunt, voorspellend van retinopatie, te bereken.

Algemene Populasie-profiel

Algeheel het die Afrikane konstant wyer venules, kleiner arterio-venulêre ratio en groter arteriolêre en venulêre dilatasie vertoon, as die Kaukasiërs. Afrikane het ‘n meer-patologiese kardio-metaboliese profiel vertoon. In die Afrikane is laer NT-proBNP vlakke, effens hoër BDNF vlakke en tog soortgelyke cTnT vlakke waargeneem, as in die Kaukasiërs. Die Afrikane het hoër: 24-uur-BP (hipertensiewe gemiddeld) en polsdruk, pre- en post-FLIP BP, intra-okulêre druk en diastoliese okulêre perfusie druk waardes, hipertensiewe/diabetiese retinopatie en voorkoms van “AV-nicking”, in vergelyking met die Kaukasiërs. Afrikane het ook groter afnames in 24-uur-HRV en HRV gedurende FLIP in beide tyd- en frekwensie-domein parameters vertoon, as hulle Kaukasiese eweknieë.

Resultate en Gevolgtrekkings

Die hoofresultate en gevolgtrekkings vir die drie manuskripte, soos voorberei as deel van hierdie proefskrif, is soos volg:

1. **Retinale vaskulêre reaktiwiteit tydens flikkerlig-geïnduseerde provokasie, kardiale stres en beroerterisiko in Afrikane: Die SABPA-studie**
   In Afrikane is ’n verlaagde arteriolêre kaliber en verswakte arteriolêre dilatasie tydens FLIP met hoër cTnT-vlakke geassosieer. Hulle groter retinale venulekaliber en verswakte arteriolêre dilatasie tydens FLIP is ook met laer vlakke van NT-proBNP geassosieer. Weereens, eksklusief in die Afrikane, het verhoogde kardiale stres, wyer venulekalibers en retinale arterio-venule “nicking” ’n verhoogde 10-jaar beroerterisiko voorspel. Geeneen van hierdie assosiasies was beduidend in die Kaukasiese groep nie. Verhoogde kardiale stres, hoofsaaklik toegeskryf aan die goed-gedokumenteerde SSS hiperaktiwiteit in Afrikane, mag bydrae tot verswakte miogene response. Waarneembare veranderinge in die retinale vaskulatuur mag dus dien as merkers om kardio-sistemiese en serebrale vaskulêre morbiditeite en risiko’s te identifiseer en voorspel – hierdeur is ’n brein-retina-hart skakel vasgestel.
2. **Harttempo-variasie, die dinamiese aard van die retinale mikrovaskulatuur en kardiale stres: Voorsien insig rakende die brein-retina-hartskakel: Die SABPA-studie**
   Afrikane het wyer venules en verswakte HRV tydens FLIP getoon. FLIP het beide verhoogde SSS en verlaagde parasimpatiese aktiwiteit (SDNN en rMSSD tyddomein-analises) *en* modulasie (LFnu, HFnu frekwensie analises) in hierdie bi-etniese groep ontlok. In Afrikane het ’n verlaagde HRV tydens FLIP gepaard gegaan met groter arteriolêre en venulêre response en verhoogde sistemiese vlakke van cTnT, wat impliseer dat die SSS ’n noemenswaardige effek op die gladdespier-tonus van die retinale vaskulatuur, direk of indirek, uitoefen. SSS-hiperaktiwiteit kan dus moontlik bydra tot ontwrigte retinale

outoregulering. Hierdie bevindings verteenwoordig sentrale beheer deur die brein op alle sistemies regulatoriese funksies, oor alle vaskulêre beddens heen.

3. **Lae Brein-afgeleide neurotrofiese faktor weerspieël verswakte retinale arteriolêre funksionaliteit en hoër beroerterisiko: Die SABPA-studie**

   Laer BDNF-vlakke – die mees oorvloedige neurotrofiese faktor in die retina – is in die totale groep, vergeleke met normale verwysingswaardes, waargeneem. Laer opvolg- BDNF-vlakke is egter slegs met verswakte arteriolêre response in die Afrikaan-groep geassosieer. Verswakte arteriolêre response is eksklusief in die Afrikane waargeneem. Alhoewel lae opvolg- BDNF-vlakke ook in die Kaukasiërs waargeneem is, blyk dit dat die direkte vasodilatoriese effekte van BDNF meer prominent in dié groep voorkom. Nietemin, lae BDNF-vlakke het retinopatie voorspel – ongeag ras of geslag. Weereens, in die totale kohort, is lae BDNF-vlakke met verhoogte beroerterisiko geassosieer. Verswakte retinalevat-response, asook verhoogde beroerterisiko, kan moontlik ’n verlaagde beskermende effek van BDNF in die SABPA-kohort aandui. ’n Volgehoue SSS hiperaktiewe toestand, soos in die Afrikane waargeneem, kan moontlik lei tot die afgestompte of verminderde aksie van BDNF, wat laer BDNF-vlakke tot gevolg het. Weens dié volgehoue SSS hiperaktiewe toestand, kan die direkte effek van BDNF of die vaskulêre gladdespier-selle aangepas wees, wat die arteriolêre weerstand wysig. Dit mag bydra tot versteurde neurovaskulêre koppeling asook verhoogde beroerterisiko, spesifiek in die Afrikane. Hierdie bevindings lê verder klem op die sentrale, neurogene beheer-funksie van die brein op alle outoregulatoriese prosesse, ongeag die vaskulêre bed.

Algemene gevolgtrekking

Die huidige studie het die moontlike rol van SSS-hiperaktiwiteit rakende die beheer van retinaal vaskulêre dinamika, veral in Afrikane, vasgestel. SSS-hiperaktiwiteit en die gevolglike gemodifiseerde hemodinamiese response waargeneem tydens sodanige hoë-druk omstandighede en chroniese kardiale stres mag direk bydra tot ontwrigte retinale outoregulering. Hierdie volgehoue hoëdruk-sisteem lei tot ’n afname in en uiteindelike verswakking van *miogene beheermeganismes*, en dui ook op ’n veranderde *neurogene respons* en *neurovaskulêre koppeling,* waarvan al voorafgenoemde bydra tot outoregulering. Daarbenewens, tydens ’n stressor soos FLIP, sal ’n reeds hiperaktiewe SSS verder uitgedaag word, en ’n verdere afname in HRV meebring – aanduidend van verhoogde SSS-aktiwiteit en modulasie, in ’n poging om genoegsame perfusie te handhaaf. Bestaande endoteel-disfunksie en gebrekkige gladdespier-response, wat aangedryf word deur SSS-hiperaktiwiteit en hoë-druk, sal moontlik as verswakte retinaal dilatoriese en konstriksieresponse manifesteer. Laasgenoemde dui ook aan dat die direkte effek of vaskulêre gladdespier-selle van BDNF moontlik gemodifiseer kan word, wat arteriolêre vaskulêre weerstand verswak en tot versteurde neurovaskulêre koppeling bydra. Hiérdie verhoogde, volgehoue SSS-hiperaktiwiteit (soos

herhaaldelik waargeneem in die SABPA-Afrikane) kan moontlik tot ’n verlengde retinalevat-respons lei, selfs nadat ’n stressor gestaak is – wat daarop dui dat die hervasstelling van die geskikte SSS-balans moeilik is wanneer so ’n balans chronies versteur is. As gevolg hiervan sal die outoregulatoriese kapasiteit van die retinale mikrovaskulatuur benadeel word en die risiko vir beroerte, retinopatie en moontlik vir optiesesenuwee-skade en gloukoom-skade in die SABPA-Afrikaanpopulasie verhoog.

Sleutelwoorde: simpatiese senuweestelsel hiperaktiwiteit; retinalevat-dinamika; flikkerlig-geïnduseerde provokasie; kardiale troponien T; amino-terminaal pro-B-tipe natriuretiese peptide; harttempo-variasie; breinafgeleide neurotrofiese faktor; beroerterisiko; etnisiteit; Suid-Afrika.

Summary

Title

Neural control, cardiac stress and retinal vascular dynamics: The SABPA study

Motivation

Structural and functional similarities exist between the retinal, cerebral and the coronary vasculature. Specifically, the retinal and cerebral vasculature show striking anatomical, functional and autoregulatory similarities –maintaining constant blood pressure (BP), despite changes in systemic BP. The latter ensures adequate perfusion and the preservation of blood-ocular or blood-brain barriers. However, the ability to maintain these autoregulatory capacities diminishes with increases in BP. Sympathetic nervous system (SNS) hyperactivity, in response to both chronic and acute stressors, may be a risk factor for hypertension, ischemic stroke and other cardiovascular diseases (CVD), specifically in Africans. These conditions primarily affect the microvasculature. The retina is the only location where the microvasculature's structure and function can be observed non-invasively. Great controversy exists regarding the legitimacy of external factors which may influence or determine myogenic, neurogenic and neurovascular coupling mechanisms – thereby impacting on the retinal vasculature's ability to autoregulate. Studies investigating SNS hyperactivity, markers of cardiac stress and neurotrophins' association with retinal vasculature are scarce or lacking all-together. A so-called brain-retina-heart link may be explored as a mechanistic pathway within the context of CVD, myocardial ischemia and stroke.

Aims

The primary aim of this study was to evaluate the influence of SNS hyperactivity, cardiac stress and neurotrophic factors on the retinal vasculature's structure and function within a South African, bi-ethnic cohort. This novel approach mandates a step-by-step evaluation and exploration of the brain-retina-heart link, providing a more holistic, preliminary mechanistic identification and translating to mainly stroke-risk susceptibility. The *Sympathetic activity and Ambulatory Blood Pressure in Africans* (SABPA) study was ideal in addressing these aims, as it is the only study in Sub-Saharan Africa which is designed to investigate the brain-heart link, by way of retinal vascular structure and function, cardiac stress markers, affiliated neurotrophic factors, cardiovascular and stroke risk, in a bi-ethnic cohort. Additionally, various investigations within the SABPA study have shown that the SABPA-Africans present with a SNS hyperactive status – hence this cohort is ideal to investigate the effects of SNS hyperactivity on retinal vascular structure and function.

Methodology

This study is an affiliate of the SABPA study. Participants were 409 African and Caucasian teachers recruited from the North West Province (Dr Kenneth Kaunda Educational District), South Africa, all of similar socio-economic, educational and healthcare status. All participants in phase 1 (2007 – 2008) were invited to take part in phase 2 (2011 – 2012). Of the initial 409 participants, 359 reported back for phase 2 of the study. Only participants who had participated in phase 2 were included, as no retinal vessel analyses were performed during phase 1. From these participants, n=41 poor/ unusable retinal vessel recordings, n=1 epilepsy were excluded from all analyses. Additional exclusions were made in the separate manuscripts to align with the set hypotheses of each. Groups were stratified according to ethnicity as evident by statistically significant interactions between major variables. The dynamics vessel analyses (DVA) was used to assess retinal vessel structure (central retinal arteriolar equivalent (CRAE), central retinal venular equivalent (CRVE) and arteriolar-to-venular ratio) and function (arteriolar and venular maximum dilation, arteriolar maximum constriction, time to dilate and to constrict and area-under the curve (AUC) analyses during flicker-light-induced-provocation (FLIP). Systemic cardiac stress markers measured in serum included cardiac troponin T (cTnT) and amino-terminal pro-B-type natriuretic peptide (NT-proBNP). Heart-rate-variability (HRV) parameters included gold-standard time- and frequency-domain parameters measured during FLIP. Brain-derived neurotrophic factor (BDNF) was measured in serum samples. Retinal venular widening, arterio-venous nicking, cTnT values above 4.2ng/mL, NT-proBNP levels below the age and gender-specific reference ranges and BDNF levels below 1.51ng/mL were applied to determine the University of California (UCLA) Stroke-risk score to assess increased 10-year stroke-risk probability. *A priori* co-variates generally included age, body surface area, serum cotinine, gamma-glutamyl transferase, glycated haemoglobin, total cholesterol/ HDL cholesterol ratio, tumour-necrotic factor alpha, hypertensive/diabetic retinopathy, 24-hour pulse pressure and vessel segment diameter (the latter in all DVA analyses). Additional adjustments were made for CRVE in CRAE models and vice versa. Statistical calculations included T-tests, analysis of covariance and Chi-square tests. Multiple linear regression analyses calculated independent relationships between major variables, while odds ratios (OR) determined stroke-risk probability based on the UCLA stroke-risk score, independent of *a priori* co-variates. Receiver operating characteristics (ROC) determined the optimal BDNF cut-point predictive of retinopathy.

General Population Profile

Overall, Africans persistently presented with wider retinal venules, smaller arterio-venous ratio and greater arteriolar and venular dilation in response to FLIP, than Caucasians. Africans displayed a poorer cardio-metabolic profile. Africans revealed lower NT-proBNP levels, slightly higher BDNF levels, yet similar cTnT values were observed between ethnic groups. The African group had higher:

24H-BP (hypertensive average) and pulse pressure, pre-and-post FLIP BP, intra-ocular pressure and diastolic ocular perfusion pressure values, hypertensive/diabetic retinopathy and prevalence of AV-nicking compared to Caucasians. Africans also showed a greater decrease in 24H-HRV and HRV during FLIP in both time and-frequency domain parameters, than their Caucasian counterparts.

Results and Conclusions

The main results and conclusions for the three manuscripts prepared as part of this thesis are as follows:

1. **Retinal vasculature reactivity during flicker-light-provocation, cardiac stress and stroke risk in Africans: The SABPA study**
   In Africans, a reduced retinal arteriolar calibre and attenuated arteriolar dilation during FLIP was associated with higher cTnT levels. Their larger retinal-venular calibre and attenuated arteriolar dilation during FLIP were associated with lower NT-proBNP levels. Again, exclusively in Africans, increased cardiac stress, wider venular calibres and retinal arteriovenous-nicking, predicted an increased 10-year stroke risk. None of these associations were evident in the Caucasian group. Increased cardiac stress, mainly attributed to well-documented SNS hyperactivity in Africans, may contribute to diminished myogenic responses. Hence observable changes in the retinal vasculature may serve as markers for the identification and prediction of cardio-systemic and cerebral vascular morbidities and risks – thereby establishing a brain-retina-heart link.
2. **Heart rate variability, the dynamic nature of the retinal microvasculature and cardiac stress: Providing insight into the brain-retina-heart link: The SABPA study**
   Africans displayed wider retinal venules and attenuated HRV during FLIP. FLIP elicited both increased SNS activity and decreased parasympathetic activity (SDNN and rMSSD time domain analyses) *and* modulation (gold-standard LFnu, HFnu frequency analyses) in this bi-ethnic cohort. In Africans, decreased HRV during FLIP accompanied greater arteriolar and venular responses and elevated systemic levels of cTnT, implying that the SNS exerted a significant effect on the smooth-muscle tone of the retinal vasculature, either directly or indirectly. SNS hyperactivity may contribute to disrupted retinal autoregulation. These findings may exemplify central control by the brain on all systemic regulatory functions, across all vascular beds.
3. **Low Brain-derived-neurotrophic-factor reflects attenuated retinal vascular functionality and increased stroke risk: The SABPA study**
   Lower systemic BDNF levels – the most abundant neurotrophic factor in the retina – were observed in the total cohort compared to the normal reference ranges. However, low follow-up BDNF levels were only associated with attenuated retinal arteriolar responses in the

African group. Attenuated arteriolar constriction responses were exclusively observed in the Africans. Although low follow-up BDNF levels were also observed in Caucasians, BDNF's direct vasodilatory effect appeared to be more pronounced in this group. However, low BDNF levels predicted retinopathy and increased stroke risk, irrespective of race or gender. Attenuated arteriolar constriction responses as well as stroke risk may indicate a diminished neuro-protective effect of BDNF in the SABPA cohort. Sustained SNS hyperactive state (supported by decreased heart rate variability, during FLIP, associating with lower BDNF levels), as observed in the Africans, may lead to blunted or diminished BDNF action, resulting in lower BDNF levels. Due to a sustained SNS hyperactive state, and high pressure conditions (lower BDNF levels were associated with increased IOP in this African cohort), BDNF's direct action on vascular-smooth-muscle cells may be altered, modifying arteriolar vascular resistance. This may contribute to disturbed neurovascular coupling as well as increased stroke risk, particularly in Africans. These findings further exemplify the brain's central, neurogenic control function exerted on autoregulatory processes, regardless of the vascular bed.

General conclusion

The current study exemplifies the possible role of SNS hyperactivity, regarding the control of retinal vascular dynamics, specifically in Africans. This role is emphasized as SNS hyperactivity and the subsequent modified hemodynamic responses observed during such high-pressure conditions, chronic cardiac stress and diminished neuroprotective effects may possibly directly contribute to impaired retinal autoregulation. This sustained high-pressure system may not only lead to a decrease in and eventual diminishing of *myogenic control mechanisms*, but also indicate an altered *neurogenic response and neurovascular coupling*, all ultimately contributing to autoregulation. The presence of pre-existing SNS hyperactivity, and additional SNS activity provoked during FLIP may further increase SNS activity and modulation. During a stressor, like FLIP, an existing hyperactive SNS (as is repeatedly evident in the SABPA Africans) will further be challenged, thereby causing further reduction in HRV indicating greater SNS activity and modulation, in an attempt to maintain adequate perfusion. Existing SNS hyperactivity-driven, pressure-induced endothelial dysfunction and impaired smooth-muscle responses may manifest as attenuated retinal vessel dilatory and constriction responses. The latter also indicates that BDNF's direct action on vascular smooth-muscle cells might be altered, attenuating arteriolar vascular resistance and contributing to disturbed neurovascular coupling. This increased, sustained SNS activity/modulation may lead to a prolonged vessel response, even after the stressor has ceased, indicating difficulty in re-establishing proper SNS balance, once this balance has been chronically disturbed. As a consequence the autoregulatory capacity of the retinal microvasculature may be impaired and the risk for stroke, retinopathy and possibly glaucoma and optic nerve damage will be increased within the SABPA African population.

Key words: Sympathetic nervous system hyperactivity; retinal vessel dynamics; flicker-light-induced-provocation; cardiac troponin T; amino-terminal pro-B-type natriuretic peptide; heart-rate-variability; brain-derived neurotrophic factor; stroke risk; ethnicity; South Africa

Preface

The entirety of this thesis is written in article format and comprises three peer-reviewed original research manuscripts. *Chapter 1* consists of a comprehensive literature overview which covers retinal microvascular structure, function and homology with other microvasculature beds. This chapter also includes a critical revision regarding retinal blood flow regulation, and the possible influence of cardiac stress, sympathetic hyperactivity and neural growth factors. *Chapter 1* also includes the aims and hypotheses, for the entire study, sub-divided into each manuscript, followed by references in accordance with the Vancouver style. The three manuscripts comprise *Chapters 2, 3* and *4*, including their respective abstracts, introductions, methods, results, conclusions and preferred referencing formats of each specific peer-reviewed journal's guidelines. *Chapter 5* consists of the main findings and conclusions, as well as the study limitations and recommendations for future research. Please note that throughout this thesis, black South Africans are referred to as Africans, whilst white South Africans are referred to as Caucasians.

Artistic images, representations, diagram descriptions and captions of images were all created by the candidate, A Wentzel, using the graphic design software Autodesk Sketchbook® and Microsoft® PowerPoint. Only parts of the figures (Figures 1.7 (heart) and 1.10 (brain outline)) were edited from figures obtained, with permission, from the certified medical art website, SERVIER®, which provides images free to be used, without copyright. All graphs were created using Microsoft® Excel and GraphPad Prism® computer software. Tables and Figures were notated by using Arabic numerals, appearing consecutively, related to the respective chapter of the thesis.

All manuscripts have been submitted to peer-reviewed journals for publication. The first article titled: ***Retinal vasculature reactivity during flicker-light-provocation, cardiac stress and stroke risk in Africans: The SABPA study***, has been published in the journal *Translational Stroke Research (*IF 8.32*)*. Results of this manuscript were presented (talk) at the *SA Heart Congress 2018*, with the abstract published in the *SA Heart Journal* of the corresponding year. The second article's rebuttal titled: ***Heart rate variability, the dynamic nature of the retinal microvasculature and cardiac stress: Providing insight into the brain-retina-heart link: The SABPA study***, has been submitted to the journal *Eye* with an IF of 3.02 (#EYE-18-1431). Partial results of this manuscript were presented (talk) at the *SA Heart Congress 2018*, with the abstract published in the *SA Heart Journal* of the corresponding year. Partial findings in this manuscript have also been presented (poster) at the 3rd European Society of Microcirculation and European Vascular Biology Organisation (ESM-EVBO) 2019 conference in Maastricht, The Netherlands. The third manuscript, titled ***Low Brain-derived-neurotrophic-factor reflects attenuated retinal vascular functionality and increased stroke risk:***

The SABPA study, is currently under rebuttal at the journal *Translational Stroke Research* (IF=8.32), and was assigned manuscript number for review (TRSR-D-19-00144). Partial findings were presented (poster) at the 3rd ESM-EVBO 2019 conference in Maastricht, The Netherlands.

The promoter and co-promoters agreed on co-authorship in all three manuscripts, and gave consent for the use of these manuscripts as part of the final thesis. The candidate was, however, solely responsible for literature searches, all initial statistical analysis, interpretation of all results, as well as planning and writing of the three manuscripts and the entire thesis. The candidate also contributed to collection and interpretation of data in the prospective African-PREDICT study, cross-sectional phase of the Examine YOUTH study, as well as performed biochemical and statistical analyses on the SABPA study (please refer to *Postgraduate Student Skills Acquirement form*).

Author's Statement

Each author's contribution to the SABPA study, each manuscript and the thesis in its entirety were as follows:

Ms A Wentzel	*Main author* – Responsible for the initial planning, conceptualisation and scientific proposal and ethical application of this doctoral study. Data analyses, statistical computation, interpretation of results, designing all figures, and writing of all manuscripts and the thesis in its entirety were also the responsibilities of the candidate.
Prof L Malan	*Promoter* – Principle investigator of the SABPA study, thus responsible for the study design, data collection and analyses. Supervised the planning and writing of each manuscript and the entire thesis. Provided guidance, support and expertise intellectual input.
Prof R von Känel	*Co-promoter* – Provided critical feedback, as well as clinical expertise and research input to all manuscripts, as well as the entire thesis.
Prof NT Malan	*Co-promoter* – Assisted in the design of the SABPA study, data collection and analyses. Assisted in the planning and writing of each manuscript and critically evaluated the entire thesis. Provided guidance, critical feedback and expertise intellectual input for all written material.
Prof W Smith	*Assistant-promoter* – Assisted in the collection and analyses of SABPA data. Provided critical feedback, as well as expertise research input to all manuscripts and the thesis in its entirety.

Hereby a statement from all co-authors validating their actual contribution to the study, thesis and each associated manuscript, thereby granting their permission that all three manuscripts may form part of this thesis:

"I hereby declare that my role as signified above is a true representation of my contribution to this study and/or thesis and associated manuscripts. I approve of these manuscripts and give my consent that these three manuscripts may be published and form part of this thesis for the degree Philosophiae Doctor of Ms Annemarie Wentzel."

Prof Leoné Malan

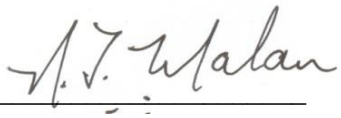
(RN, HED, PhD)

Prof Dr Roland von Känel

(MD)

Prof Wayne Smith

(PhD)



Prof Nicolaas T Malan

(DSc)

Ms Annemarie Wentzel

(BSc Hons, MSc)

POSTGRADUATE STUDENT SKILLS

STUDENT NAME: Annemarie Wentzel	**Tick if accomplished**	
Undergraduate teaching *(indicate number of courses)* Physiology for Pharmacy (FLPX113) Introductory Physiology (FLGX113) Neurophysiology (FLGX325)	N = **3**	**X**
Optional: Clinical Pharmacology course *(16 credit module)*	**X** (90% ave)	
Optional: Honours student mentorship *(indicate number of students)*	N = **2**	
Ethical consent: *Sub-study application under Umbrella-study*	N = **3**	
Obtained and interpreted medical history, medication status: *Socio-economic (medical aid access, education; job), marital, family history, health and cardio-metabolic incidents/events; medications*	**X**	
Dietary habits questionnaire	**X**	
Good Clinical Practice (GCP) course: *Year obtained*	**X (2017)**	
[1]Observed collection/[2]Interpreted psychosocial battery measures: *Measures with known heritability: Life orientation, Personality*	[1] **X**	[2] **X**
Predictors of developing/worsening hypertension: Coping, Depression, Cognitive distress	**X**	
Moderating effects of the environment: Fortitude, Mental Health, Self-regulation, Job stress	**X**	
[1]Observed/[2]Interpreted anthropometry measurements *Height, Body mass, Waist circumference, Physical activity*	**X**	
[1]Cardiovascular assessments, [2]download and [3]interpretation of data *Resting Blood Pressure [Riester CE 0124® & 1.3M ™ Littman® II S.E. Stethoscope 2205]*	**X**	
**Finometer [Finapres Medical Systems®]*	**X**	
12-lead resting ECG [NORAV PC-ECG 1200®]	**X**	
24 ambulatory BP & -ECG [Cardiotens® & CardioXplore, Cardiovisions 1.19®, Meditech]	**X**	
Pulse Wave Velocity and Pulse Wave Analysis [Sphygmocor EXCEL, AtCor]	**X**	
Laboratory skills (sample handling and analyses) *24h Urine/blood/saliva/hair: [1]collection/[2]sampling/[3]aliquoting/[4]waste material*	[1] **X** [3] **X**	[2] **X** [4] **X**
Rapid tests (cholesterol, glucose, urine dipstick and blood type)	**X**	
Laboratory analyses of samples (ELISA, RIA, COBAS Integra, E411)	**X**	
Whole blood HIV status *[PMC Medical, Daman, India; Pareekshak test, BHAT Bio-Tech, Bangalore, India]*	**X**	
[1]Accomplished training & [2]measuring of ultrasound Carotid Intima Media Thickness (CIMT) *[Sonosite Micromaxx®, SonoSite Inc., Bothell, WA]* **[3]Retinal Vessel Assessment, [4]Data download & Interpretation** *(Imedos®)*	[1] **X** [3] **X**	[2] **X** [4] **X**
Statistical analyses *[1]Normal distribution & T-tests, [2]General linear models, [3]Multiple regression analyses* *[4]ROC analyses; [5]Prospective data analyses and risk prediction*	[1] **X** [4] **X**	[2] **X** [3] **X** [5] **X**
Successful grant/funding application/s: *NRF[1]/MRC[2] South Africa*	[1]N = **2**	[2]
Publications: *Prepared, submitted, handled rebuttal of manuscript in a peer-reviewed journal*	N= **5**	
Publications: *Served as a reviewer for a peer-reviewed journal*	N= **1**	
Conference meetings: *[1]National, [2]International [3]oral/[4]poster presentation*	[1]N= **4** [3]N= **5**	[2]N=**3** [4]N= **3**

N=number; *Inclusive of sympathetic nervous system (SNS) responses (acute mental laboratory stressors e.g. cold pressor & colour-word-conflict)

Prof L Malan (RN, HED, PhD)
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Nomenclature

Symbol/Abbreviation	Description
α	Alpha
β	Beta
%Δ	Percentage Change
mmHg	Millimetre of Mercury
γ-GT	Gamma Glutamyl Transferase
CRP	High sensitivity C-reactive Protein
ABPM	Ambulatory Blood Pressure Monitoring
ANCOVA	Analysis of Covariance
ANS	Autonomic Nervous System
AV	Arteriovenous
AVR	Arteriolar to Venular ratio
AUC	Area Under the Curve
BDNF	Brain-derived Neurotrophic Factor
BMI	Body Mass Index
BP	Blood Pressure
BSA	Body Surface Area
CAD	Coronary Artery Disease
CI	95 % Confidence interval
CNS	Central Nervous System
CO	Cardiac Output
cTnT	Cardiac Troponin T
CRAE	Central Retinal Arteriolar Equivalent
CRVE	Central Retinal Venular Equivalent
CVD	Cardiovascular Disease
C_{wk}	Windkessel compliance
DBP	Diastolic Blood Pressure
DOPP	Diastolic Ocular Perfusion Pressure
DPPIV	Di-peptidyl Peptidase 4
DVA	Dynamic Vessel Analyser
ECG	Electrocardiogram
et al	Et alia “and others”
F	Female

FLIP	Flicker Light Induced Provocation
HbA_{1c}	Glycated Haemoglobin
HDL	High Density Lipoproteins
HF	High Frequency
HR	Heart Rate
HRV	Heart-Rate-Variability
HRVti	Heart-Rate-Variability Triangular Index
IDE	Insulin Degrading Enzyme
IOP	Intra Ocular Pressure
L	Litre
LF	Low Frequency
LVEF	Left Ventricular Ejection Fraction
LV	Left Ventricle
LVH	Left Ventricular Hypertrophy
M	Male
MC	Maximum Constriction
MD	Maximum Dilation
MI	Myocardial Ischemia
M/L	Media-to-lumen ratio
MU	Measuring Units
µm	Micro-metre
mL	Millilitre
mm	Millimetre
mV	Milli-Volt
N	Number of Participants and/or Events
NEP	Nor-endopeptidase
ng	Nano-gram
NO	Nitric oxide
NT-proBNP	N-terminal pro-B-types Natriuretic Peptide
NVU	Neurovascular Unit
NWU	North-West University
OR	Odds Ratio
pg	Pico-gram
PNS	Parasympathetic Nervous System
PP	Pulse Pressure

rMSSD	Root Mean Squared of the Standard Deviation
ROC	Receiver Operated Characteristics
RPE	Retinal Pigment Epithelium
SABPA	Sympathetic Activity and Ambulatory Blood Pressure in Africans
SBP	Systolic Blood Pressure
SD	Standard Deviation
SDNN	Standard Deviation of NN-intervals
SDRR	Standards Deviation of the RR-intervals
SE	Standard Error of the Mean
SMC	Smooth-Muscle Cell
SNS	Sympathetic Nervous System
SV	Stroke Volume
TEE	Total Energy Expenditure
TNF-α	Tumour Necrotic Factor alpha
TPR	Total Peripheral Resistance
UCLA	University of California
U/L	Units per Litre
WHO	World Health Organisation
W/L	Wall-to-lumen ratio

Chapter 1

Literature overview, aims and hypotheses

Literature Overview, Aims & Hypotheses

1. General introduction

Challenges, threats or stressors are not fatal, but how we respond to them, mentally and physically, may be. Residents of the 21st century are faced with severely challenging demands and may experience a state of increased mental strain. This results in an attempt of the body to adapt physiologically (1). Consequently arrays of biochemical pathways are activated to accommodate acute as well as chronic stressors. These result in both transient and permanent structural and functional modifications to the cardiovascular system, modifications that may already be evident at microvascular level (2). Failure to successfully adapt or respond to physical and mental stressors is associated with an increased prevalence of hypertension, followed by microvascular rarefaction and contributes to ischemic heart disease and stroke susceptibility (1, 2).

Research spanning nearly 30 years has consistently shown that Africans have a greater risk profile for the development of hypertension, metabolic syndrome and ischemic heart disease (also referred to as coronary artery disease or CAD) than their Caucasian counterparts (3-13). Sub-Saharan African men also present with an increased stroke risk, as recently reported by Hamer and co-workers (14). This risk profile has mainly been attributed to the increasing urbanized lifestyle, intensifying the psycho-social stress experienced by Africans (3, 11). Indeed acute stressor-induced reactivity differences were evident between Africans and Caucasians. Malan and co-workers have shown that Africans that reside in urban environments tend to indicate increased hypertension prevalence and exhibit α-adrenergic vascular responses, whilst their Caucasian counterparts present a central cardiac β-adrenergic response accompanied by normal blood pressure (BP) values (3). Sympathetic nervous system (SNS) hyperactivity has been identified as one of the primary contributing factors of cardiovascular disease (CVD) and ischemic stroke development, especially in mentally stressed individuals (15).

Mental stress has also been linked to transient dysfunction in the microvasculature (16). Indeed, the SNS is the major motor output pathway for any perceived stressor (6, 17-19). Whether a stressor is experienced in everyday life, or artificially recreated in a laboratory, the SNS response remains the same (20). Numerous studies conclusively recognise SNS hyperactivity, in response to both chronic and acute stressors, as a risk factor for the development of hypertension, ischemic stroke, myocardial infarction and other CVDs – all of which primarily originate at microvascular level (17, 21-23). Such SNS hyperactivity may initially be a homeostatic attempt to maintain adequate central and peripheral perfusion pressures. However, if sustained, it may have detrimental effects (22). Demographic factors such as age, ethnicity, as well as psychological aspects including perception and previous

experiences, determine and shape this highly integrated stressor-induced SNS reactivity and lasting effects of sustained SNS hyperactivity (1, 18, 19). Hence it is imperative to identify, elucidate and characterise the possible role SNS hyperactivity plays in microvascular function, and translate this role to cardiac stress and neurotrophic factors – specifically within the context of retinal blood flow regulation.

The retina provides an easy, non-invasive access to the microvasculature both for research scientists and clinicians. Dynamic vessel analyses (DVA) allows for the online measurement of retinal vessel calibres and changes therein during flicker-light-induced provocation (FLIP) (6, 24-29). The manner by which these changes occur may indicate how other microcirculatory systems (neuro-microvasculature and coronary microvasculature) react when faced with increased metabolic demands such as during mental or physical stress (6). However, whether FLIP itself is a stressor that elicits a SNS-driven response, and whether the consequences of sustained SNS hyperactivity may modify retinal vessel responses, remains to be determined.

Due to increased psychosocial stress, CVD and stroke risk, particularly exhibited by Africans, it is of utmost importance to identify reliable markers and propose physiological mechanisms that can identify pre-clinical risk, especially in vulnerable individuals. Research exploring such markers, their inter-relationships and application in risk stratification is insufficient. This study may thus shed light on the SNS influence on retinal vascular dynamics – a relationship that may have translational clinical value. A novel approach mandates a step-by-step evaluation and exploration of the brain-retina-heart link, providing a more holistic method to preliminary mechanistic exploration. The relationship between SNS-associated systemic cardiac stress markers (cardiac troponin T and amino-terminal pro-B-type natriuretic peptide) and myogenic control mechanisms of the retinal vasculature must be investigated to establish the retina-heart link. The controversial topic, regarding stressor-evoked SNS activity (heart-rate-variability) on retinal vessel dynamics, should subsequently be addressed to evaluate one aspect of neurogenic control of retinal vasculature. Finally, a feature of the neurovascular coupling aspect of neurogenic control should be assessed. Here the most abundant neurotrophic factor in the retina, which is directly linked to SNS activity, brain-derived neurotrophic factor's impact on retinal vasculature dynamics, should be evaluated.

2. The Human Retina

"*Ut imago est animi voltus sic indices oculi*" – The face is the picture of the mind, as the eyes are its interpreter. These words were spoken by Cicero between 106 and 43 BC, yet countless philosophers, politicians, writers and speakers have quoted numerous versions of this phrase. However, today we know that quantifiable, scientific evidence substantiates investigations of the eye to be useful in

exploring the brain, but also the cardiovascular system and affiliated diseases. Indeed, the eye *is* the window to the mind and heart.

3. Anatomy of the Retina

During embryonic development the retina and optic nerve develop from the diencephalon, and the retina therefore is considered an extension of the diencephalon (30-33). A structure mirroring the complexity of the central nervous system, the anatomical organization of the retina can be generally described in topographical, histological and cellular fashion (**Figure 1.1**). Yet, as is the aim of the current study, special consideration will only be given to the human retinal vasculature and its associated cellular organisation.

4. The retinal vasculature: a surrogate for brain microcirculation

The retina is the neurosensory part of the human eye, thus the part that reacts to external, visual stimuli (34). The retina lines the inner surface of the eye and is a multi-layered structure depicted in **Figure 1.1**. The photoreceptors in the retina respond to visual and light stimuli, transferring these signals to the brain via the optic nerve (33). The retinal circulation comprises two systems: 1) the *uveal circulation*, supplying blood to the uveal layer of the eye, as well as the middle and outer layers of the retina, and 2) the *retinal circulation* which supplies the inner layers of the retina (32, 33). The central retinal arteriole and venule bifurcate several times to shape this intrinsic vascular network resulting in a unique structural pattern. The retina is the only part of the human body that presents the opportunity of non-invasively assessing and visualising the microvasculature, but also translates these structural and functional observations to other microvasculature beds (28-34).

The retinal and cerebral microvasculature share many physiological and morphological characteristics (32, 33). The retina and brain are tissues of unparalleled sophistication; however, both seem to have a fundamental design conundrum: they both are highly metabolically active tissues, yet lack the energy reservoirs necessary to maintain such high metabolic activity. Consequently both these tissues are dependent on second-to-second delivery of energy substrates, oxygen and glucose. Considering the regionally diverse and dynamic nature of these energy requirements, timely blood flow needs to reach the brain and retina, in sufficient amounts, depending on the situational demand.

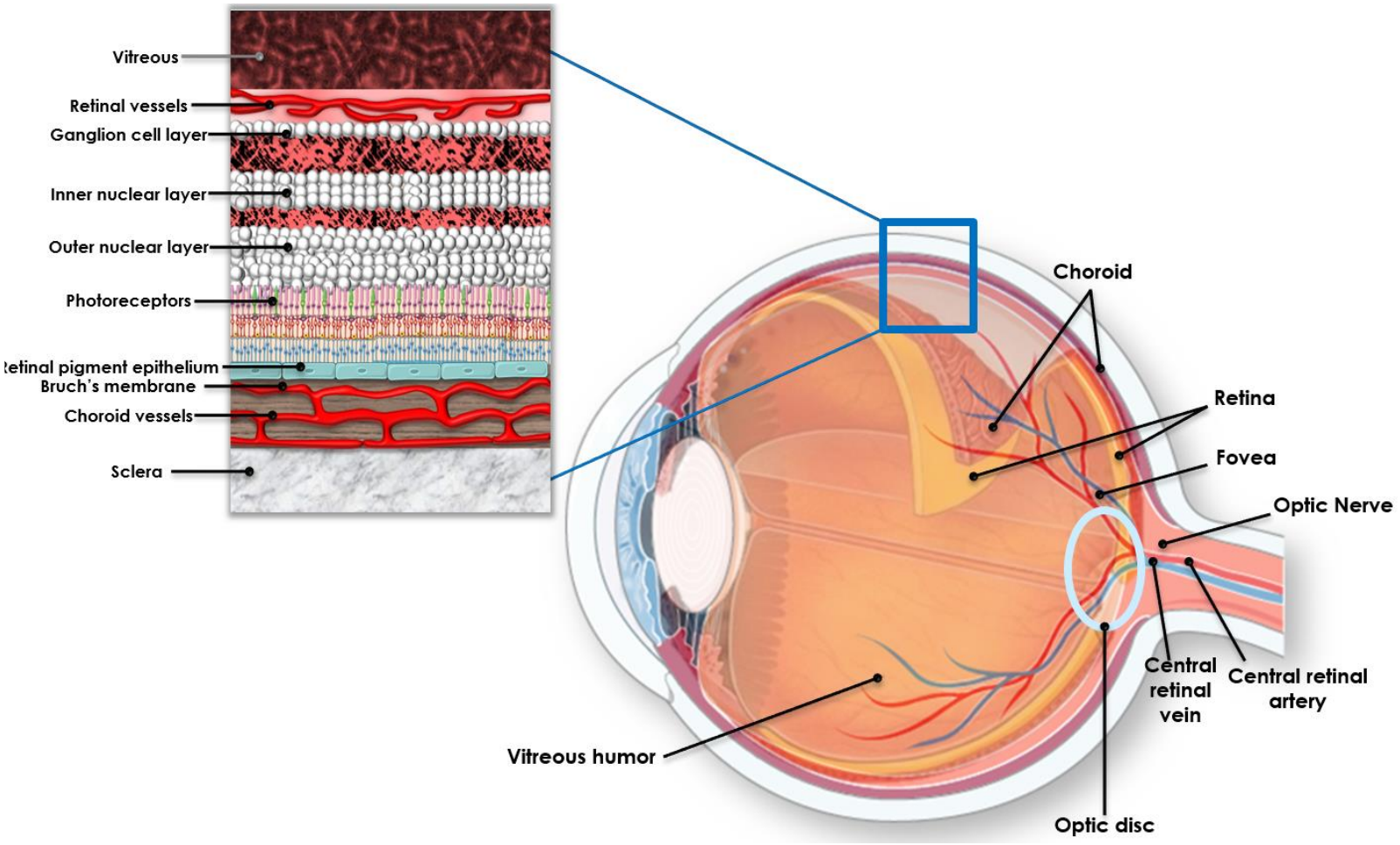

Figure 1.1: Representation of the eye, retina and retinal layers

4.1. Anatomical homology between retinal and cerebral microvasculature

The central retinal artery branches off from the ophthalmic artery virtually immediately after entering the eye and enters the optic nerve approximately 1cm behind the globe (30, 33) (**Figure 1.1**). The density of retinal arterioles and capillaries is greater in the central retina and decreases towards the peripheral retina (35-37). A similar trend is observed in the cerebral circulation, where the density of arterioles and capillaries vary depending on the metabolic demand of a particular region – for instance, white matter and sensory centres are more richly supplied than grey matter and motor centres (32).

In the retina, the smaller arterioles give rise to specific types of capillary systems: 1) the superficial nerve fibre/ ganglion cell layer served by horizontal branches and 2) deeper branches that enter the retina to create between one and four horizontal capillary layers within the inner retina, from the periphery and perifoveal to peripapillary respectively (30). Therefore the retinal circulation supplies all layers of the neural-retina except for the photoreceptor layer, the latter depends on the choroidal circulation (35-39).

It is important to note that the retinal arteries are end-arterial and consequently lack anasmotic connections (32, 33). This implies that any interruption of a retinal arteriole results in the loss of blood supply to that specific region of the capillary bed and eventual destruction of inner retinal layers (32, 40). Increased intra-ocular pressures (IOP) and subsequent inadequate perfusion will lead to retinal damage, such as is the case in hypertension (40). The retinal arteriole and venular vessels also affect one another, due to their close physical proximity. Arterial hypertension, for example, may cause a retinal artery to compress an adjacent vein, presenting as the pathological phenomenon defined as arteriovenous nicking (AV-nicking) (38).

4.2. Homology between retinal and coronary microvasculature

The coronary arteries, possessing an elastic lamina, are tailored to accommodate frequent pulsatile changes, whereas the retinal and cerebral microvasculature's structure ensures adequate perfusion. Despite this difference, recent evidence showed that comparable structural and functional similarities may exist between the retinal and coronary microvasculature (40, 42). Whether retinal vessel structural and functional properties link to traditional cardiac stress markers, remain to be investigated.

4.3. Elements of the retinal vascular unit

Neurons, glial cells and the cerebral endothelium exhibit a unique relationship as they function as a cohesive unit, similar to that of the retinal microvasculature (31, 42). This unique interaction is not

only one of the most striking similarities between the cerebral and retinal microvascular beds, but also one of the main contributors to the distinct regulation of blood flow within these vasculatures (**Figure 1.2**).

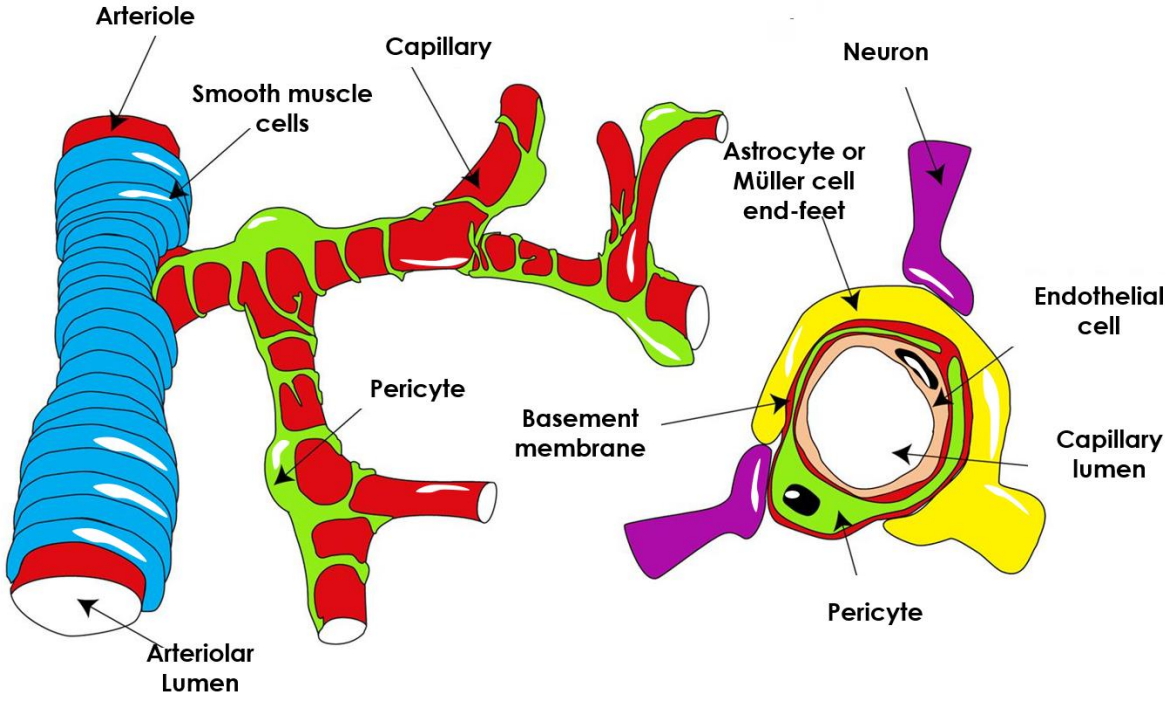

Figure 1.2: Graphical representation of the neurovascular association present in arterioles

Endothelial cells

The mechanical element of the distinctive blood-retinal barrier is formed by the tight junctional, non-fenestrated intercellular complexes between endothelial cells (31). These tight junctional processes are composed of several proteins, including transmembrane proteins (17). In both the retinal and cerebral capillaries, the endothelial cells create a single layer which surrounds the capillary lumen (31-33). The scarcity of pinocytotic vesicles and lack of fenestrations additionally support the great number of specialised carrier-mediated transport proteins present in the endothelial cells of the retinal microvasculature (32, 43). Naturally, such great numbers of carrier-mediated transport proteins will also justify the large number of mitochondria in the endothelial cells. These energy-demanding transport mechanisms create the metabolic component of the blood-retinal and barrier (44, 45). Specifically glucose and amino acid transporters are essential elements of this metabolic component, ensuring adequate transport of nutrients to these metabolically taxing tissues (44).

Vascular smooth-muscle cells and pericytes

The vascular smooth-muscle cells (SMC) surround the retinal arterioles (**Figure 1.2**). Specifically, the pial arterioles (those arterioles on the cortical surface, from which penetrating arterioles originate) and penetrating arterioles (those that have moved through the blood-retina barriers), are coated with a thick layer of SMC, essential in the control of vascular tone (31). Various pathological conditions, such as SNS hyperactivity, hypertension and increased cardiac stress, may alter the SMC's tone and contractile ability (31). Pericytes are the capillary counterparts of vascular SMC, surrounding the capillary endothelial cells (31, 32, 44, 45). Pericytes also exhibit contractile properties, and may participate in up-stream propagation of contractile responses, possibly affecting the diameters of the arterioles preceding the capillaries (31, 45).

Basement membrane

The main function of the basement membrane is to provide structural support to the microvasculature (32, 43). Yet it also plays a fundamental role in endothelial function, specifically in response to vasodilatory agents such as nitric oxide (NO) (43). The retinal and cerebral basement membranes are the site of many pathological conditions, including β-amyloid deposition in Alzheimer's (33) as well as thickening in SNS hyperactive and hypertensive mammals (32, 33). Pathological thickening of the retinal microvasculature basement membrane occurs as an integral feature of diabetic and hypertensive retinopathy (46).

Glial cells

The retinal and cerebral microvasculature is surrounded by a number of perivascular end-feet, also known as astrocytic processes (31, 47). Numerous *in vitro* studies showed that these astrocytic processes, in addition to providing structural support, also play an essential role in the production, development and maintenance of healthy endothelial cells (47). Systemic SNS hyperactivity may also initiate morphological and functional changes in the glial cells, impacting on the endothelium and SMC function (31). Indeed, retinal astrocytes, as well as Müller cells, influence the vascular endothelial cells and directly alter the SMC's contractility, via humoral factors, such as brain-derived neurotrophic factor (BDNF) (33, 48). Retinal astrocytes and Müller cells stimulate angiogenesis via the release of growth factors during hypoxia and metabolically demanding situations (48-50), possibly directly contributing to blood-flow regulation during metabolically taxing scenarios. The latter may also link glial cell function to SNS activity (48).

4.4. Retinal imaging

The fascination with the retina is no new venture. Since the conception of the ophthalmoscope, retinal examination became routine for ophthalmologists. The first image of the retina (fundus image) was published in 1853 by the Dutch ophthalmologist Van Trigt, after several modifications that led to a reinvented ophthalmoscope from 1851 (51). However, the original principles of the ophthalmoscope was developed by the Czech researcher JE Purkynje and his preliminary sketches of the retinal vasculature were already published in 1923 and re-published in 1939 (52). In 1910 the Nobel Prize was awarded to the renowned Swedish ophthalmologist and optician Allvar Gullstrand for his development of the fundus camera (54). Gullstrand's principle of fundus imaging and the schematic descriptions of the dimensions of the eye (**Figure 1.3**) are still applied today.

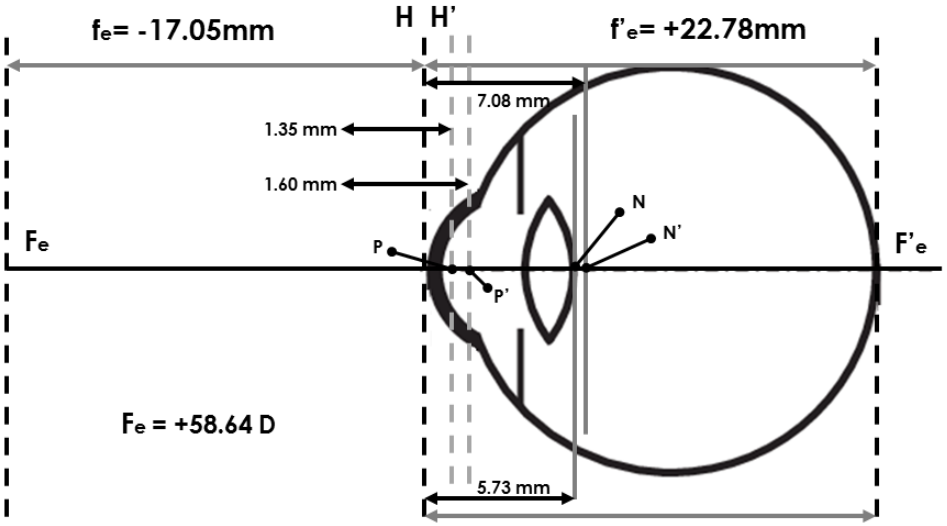

Figure 1.3: The Gullstrand eye. Where: Fe, frontal focus point; Fe', focus point where collimated beams come to focus on the fovea; P, principal plane; P', derivative of the principle plane (unit magnification) – used to generate an effective lens; N, Nodal point (front); N', rear nodal point – a ray that passes through the front nodal point at a given angle, leaves the rear nodal point at the same angle; H, initial refraction surface of the cornea; H', secondary refraction surface; D, dioptres

Fundus imaging creates an optical pathway between the camera and the retina. An internal electronic flash, sharing an optical path with a reflex mirror, ensures maximum illumination of the retina. Due to the rapid technological advances, digital imaging technology was rapidly adapted for retinal imaging. Instant digital imaging feedback, adjustable exposure settings and camera alignment together with the continued development and improvement of imaging hardware and software, ensures that digital retinal imaging may be frequented as a research and clinical tool. Digital imaging of retinal vessels has become increasingly common over the past two decades and the development of progressively advanced and sophisticated techniques, such as inducing reactive changes in retinal vessels to assess functionality, have created new and exciting research and clinical avenues.

Various retinal imaging techniques and modalities exist, including the classic ophthalmoscope, retinal camera, scanning laser ophthalmoscopy, stereo retinal photography, fluorescein angiography, indocyanine green angiography, optical coherence tomography and retinal vessel analyses, specifically the dynamic vessel analyser.

Dynamic vessel analyses (DVA) is a relatively new method that allows for the online measurement of retinal vessel calibres and changes therein during and following flicker-light-induced provocation (FLIP) (24-29). This allows for the analyses of the dynamic nature of the retinal microvasculature and provides a unique window to non-invasively study the condition of the microvasculature and endothelial function (25, 54, 55). The manner in which these changes occur may indicate how other microcirculatory systems (neuro-vasculature and coronary microvasculature) react when faced with increased metabolic demands such as during mental or physical stress. Due to the homology between the retinal and cerebral vasculatures, and recently suggested, the coronary microvasculature, changes and attenuated reactivity of the retinal vessels may reflect similar changes in other microvascular beds (26-28, 32).

Retinal imaging and clinical implications

Structural changes

It is important to note that although it is customary to generally refer to retinal structural changes, in truth it is the diameter of the erythrocyte column that is measured – thus essentially, it is lumen narrowing that is defined as structural changes (56). Pathological conditions such as systemic hypertension, coronary heart disease, diabetes mellitus, cerebrovascular disease (including ischemic stroke) and depression have all been associated with structural modifications of the retinal vessels (29, 56-59). These conditions may also be preceded by sustained SNS hyperactivity (8, 22, 58). Structural modifications include arteriolar narrowing, wider venules, a decreased arteriovenous ratio (AVR) and AV-nicking (60-63). Four grades of retinal vascular structural changes are proposed, specifically in hypertensive patients, 1) focal or general arteriolar narrowing; 2) AV-nicking; 3) flame shaped haemorrhages and hard exudates and 4) papilledema (64). In diabetic patients, similar classifications are considered, with the addition of micro-aneurysms, cotton wool spots and abnormal vessel growth (neovascularisation) (64). Arteriolar narrowing and AV-nicking are observed more frequently within epidemiological settings, and are considered less severe. However, AV-nicking, is a recognized phenomenon related to arteriosclerotic thickening of the small vessel walls, and directly associated with a higher risk for ischemic stroke (65). Subsequently, the clinical and predictive relevance of these mild degrees of retinopathy are questioned, as these changes seem to be largely non-specific arteriolar changes (29, 60). However, evidence from cross-sectional as well as longitudinal investigations reveal an independent association between retinal arteriolar narrowing

and elevated blood pressure (BP) (59, 61-63, 66). Wong and colleagues (63) also found that the combined exposure to a high BP system and narrower retinal arterioles was associated with a higher risk of hypertension than each of these effects individually. A smaller AVR, mainly attributed to narrower arterioles, may precede arterial hypertension in patients who were initially normotensive (66, 67). Arteriolar narrowing and wider retinal venules are also associated with an increased risk for ischemic stroke, irrespective of systemic hypertension (59, 62). Indeed, this notion supports the occurrence of microvascular rarefaction, which refers to the structural and functional remodelling of arterioles and venules in response to sustained high BP and ultimately involves the destruction of blood-vessels (68). Such rarefaction not only increases the peripheral vascular resistance, but also directly results in ischemic target organ damage, such as myocardial infarction and ischemic stroke (68).

Preliminary evidence suggests that structural retinal vascular changes may serve as an early indicator of the severity and progression of coronary artery disease (CAD) (69). This might possibly be due to the coronary perfusion deficits evident in CAD, contributing to microvascular rarefaction and advanced ischemia. Indeed, patients with progressed CAD present with pathologically sparse microvascular densities (70). This also supports the link between stroke and structural changes in the retinal vasculature. Recent evidence indicated that wider retinal venules were associated with increased stroke risk within an epidemiological setting (22). This indicates that dilated veins, opposed to narrower arterioles, were linked to worse vascular prognosis. Contrary to popular opinion, veins are not passive vessels, but also actively adapt to vascular needs (29). However, additional investigations are needed to explore the mechanisms involved, ideally in combination with other risk markers. This may then serve as an investigative and mechanistic tool to describe cardio- and cerebral vascular risks, thereby establishing a more defined brain-retina-heart link.

Ethnic-specific structural changes

Recent evidence within the Sympathetic activity and Ambulatory Blood Pressure (SABPA) study has revealed ethnic differences regarding structural retinal vascular components (8, 22). Indeed, the African group persistently exhibited an increased CVD and stroke-risk profile, with greater SNS hyperactivity, subsequent to hypertension prevalence and increased cardiometabolic risk (6-12, 22, 71-73). Yet, one could argue that this observation may be due to the poorer cardiometabolic profile observed in the African group and if these profiles were matched, such ethnic differences might disappear. This does, however, not negate the constantly reported ethnic-disparities observed between Africans and Caucasians within this cohort. The SABPA African men presented with narrower retinal arterioles, wider retinal venules, AV-nicking and greater retinopathy prevalence than did their Caucasian counterparts (8, 22). However, the retinal vessels dynamics within these ethnic groups and its relation to cardio-cerebral risk remains to be explored.

Functional changes

The microvascular morphology, also relates to the retinal vessels' ability to make functional changes correlating with local demand or systemic alterations. Indeed, one could argue that the functional changes precede the observed structural changes that occur in the retinal vasculature during pathological conditions. However, functional and structural changes are not necessarily concurrent, since a vasculature that appears structurally normal may present with functional impairment (50). Flicker-light-induced-provocation (FLIP) or flicker-light stimulation triggers a dilatory and compensatory constriction response of the retinal vasculature (26). The changes in retinal vessel diameters to FLIP have also been identified to be reproducible, and support the existence and influence of neurovascular coupling in the human retina (26). Attenuated retinal vessel responses are indicative of and associated with various pathological conditions.

In hypertensive patients attenuated retinal arteriolar, but not venular, responses to FLIP were observed (74). SNS hyperactivity is common among hypertensive patients, and such a compromised, high-pressure system may contribute to attenuated retinal vessel responses. In patients at high risk for the development of coronary artery disease, focal arteriolar narrowing and AV-nicking was associated with a reduced FLIP retinal arteriolar dilation (23). Conzen and co-workers reported a prolonged latency of retinal arteriolar dilation, vasoconstriction and impairment of the retinal neurovascular coupling unit in patients who suffered subarachnoid haemorrhages (75). Indeed, findings by Kotliar et al (28) indicated that a retinal arteriolar reaction was increased and dilation was delayed in patients suffering from Alzheimer's disease. They concluded that this possibly indicates that retinal neurovascular coupling might be compromised with differentiating alterations across the spectrum of Alzheimer's (28). This may also support attenuated vessel responses relating to an ischemic stroke risk (2).

Attenuated microvascular responses have been reported in pre-diabetic and type 2 diabetic patients (76-78). However, such retinal vascular dysfunction may contribute to the pathogenesis of diabetic retinopathy, independent of endothelium dependent mechanisms. Indeed, although retinal autoregulation was impaired in diabetic patients, retinal responses to exogenous nitric oxide (NO) (a potent endothelium dependent vasodilator) were similar to those of healthy controls, implying maintained endothelium sensitivity to NO in diabetes (76). Hence other factors, including SNS hyperactivity, common in patients with diabetes, might play an important role in altered vaso-activity of retinal vessels.

Consequently flicker-light-induced changes in retinal arteriolar and venular diameters, may provide essential information on the condition of the vascular endothelium, myogenic mechanisms,

neurogenic processes and the neuro-vascular unit. All of these factors contribute to and are responsible for some aspect of retinal blood flow regulation.

5. Regulation of retinal blood flow

As previously stated, the retinal, cerebral and cardiac tissues are metabolically highly active tissues which require a constant, uninterrupted supply of oxygen and glucose. This implies constant blood flow and adequate pressure to ensure sufficient perfusion. Therefore highly sophisticated regulatory mechanisms must ensure such constant delivery of nutrients and oxygen, despite changes in a broad range of local (increased metabolic demand, hypoxia or hyperglycaemia) as well as external factors such as changes in systemic blood pressure due to changes in metabolic requirements. Yet, how the afore-discussed components feature in these regulatory mechanisms, specifically relating to SNS-driven systemic pressure changes and central markers, are poorly understood and under-investigated.

This creates a new avenue of investigative possibilities and discussions. Even though the local process of control, also known as autoregulation, is a distinct property of the retinal, cerebral and coronary microvascular systems, the perfusion pressures of the retinal, cerebral and coronary microcirculations relate to and are dependent on changes in systemic blood pressure. This may additionally support why cardio-cerebro-systemic pathologies, such as hypertension and stroke, are reflected by retinal structural and functional changes (2, 8, 63, 75).

Naturally, the heart creates, regulates and sustains systemic blood pressure as it is the central pressure provident. All hemodynamic changes that occur in the arterial tree are thus mainly influenced by pressure changes governed by the heart (79). In the microvasculature the exceptional physiological mechanism of autoregulation exits, seemingly regulating local BP independent of systemic BP changes (80). Even in the coronary microvasculature, the medium-sized arterioles' diameters are governed by autoregulatory processes. However, alteration in perfusion pressures will modify the myogenic response and endothelium-mediated regulation, followed by metabolic (cerebral, coronary and retinal) and neurogenic (cerebral and retinal, coronary uncertain) control (79). The retinal microvasculature perfusion pressure is related to systemic blood pressure and intraocular pressure by the relationship:

Ocular blood flow = [Mean arterial blood pressure – Intraocular pressure]/vascular resistance

This relationship can be applied to the cerebral and even cardiac microvasculature. It is clear from this equation that the vascular resistance is to be altered in order to maintain constant retinal blood flow over a wide range of systemic BPs. The ideal intra-ocular pressure (IOP) ranges from 12-20mmHg of the mean arterial BP (32), with retinal detachment occurring at 30-50mmHg (17).

Vascular resistance can be modified when changes occur in vessel diameters, predominantly via alterations in the vascular smooth-muscle tone of the retinal arterioles, glial cell influence and pericytes (31, 32, 80). Generally, endothelium dependent, myogenic, neurogenic and metabolic processes are assumed to be involved in the process of autoregulation of the retinal microvasculature (17, 81).

5.1. Endothelium-dependent and myogenic control of retinal blood flow

An impaired endothelial function, although not pathognomonic, is a characteristic of conditions leading to myocardial infarction and ischemic stroke (2, 29). Such functional impairment precedes the development of the morphological vascular changes, described in pervious sections, likely as a protective measure and attempt to maintain homeostasis. Local retinal arteriolar dilation has been shown to be endothelium and NO-dependent (82, 83), yet seemingly independent of direct SNS influence (29, 32). NO is essential for maintaining arteriolar and venous tone (83), and a key player in the hyperaemic response to FLIP (22). Indeed, attenuated arteriolar dilation was observed in patients suffering from atherosclerosis, hypertension, type 2 diabetes and dyslipidaemia (2, 29). In all cases the vessel dilatory capacities were improved by the respective treatment (2, 29). NO-insensitivity has also been observed in patients, post-stroke (2). Contrary-wise, although retinal autoregulation was impaired in diabetic patients, retinal responses to exogenous nitric oxide (a potent endothelium dependent vasodilator) were similar to those of healthy controls, implying maintained endothelium sensitivity to NO in diabetes (79). This may imply that other factors coincide to alter endothelial NO-sensitivity (e.g. SNS hyperactivity, changes in systemic blood flow and perfusion pressures). So any element that may alter or influence the endothelium's secretion of and response to NO (whether eNOS, iNOS or nNOS) will adversely affect retinal vessel dilation.

Aside from hyperaemic governance of vessel reactivity, the local process of control, autoregulation, is a distinct property of the retinal, coronary as well as cerebral microvascular systems (84). However, autoregulation is not absolute, nor undeviating. When systemic BP increases with approximately 40% or greater, autoregulation is negated and retinal blood flow will increase as myogenic control will dissipate (32). Myogenic control mechanisms are the capacity of both the vascular SMCs and the pericytes of the microvasculature to contract in response to an increase in transmural pressure (31, 85). This is also known as the Bayliss effect (**Figure 1.4**) and is essentially responsible for decreasing the blood flow through a blood vessel after a systemic increase in BP (40, 86). High systemic BP, such as evident in SNS hyperactivity, leads to the decrease in and eventual diminishing of the myogenic control mechanism, ultimately contributing to autoregulation (86). This mechanism is seemingly independent of SNS influence. However, the effect of changes that occur in the rest of the arterial tree due to central input cannot be ignored. Indeed, increased SNS activity, drive and tone, accompanied by systemic hypertension (21), causes a shift in the autoregulatory range of the

microvasculature (**Figure 1.5**) – thereby, detrimentally altering perfusion pressures and flow in retinal, cerebral as well as cardiac tissues.

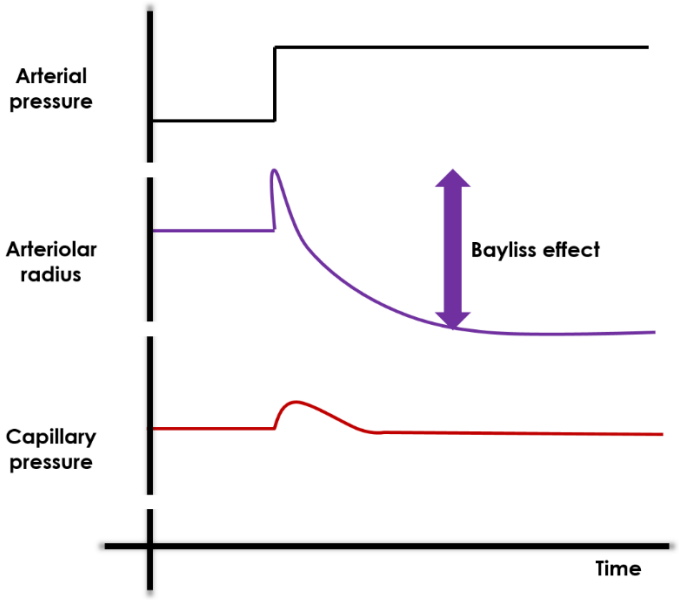

Figure 1.4: The Bayliss effect or myogenic control mechanism

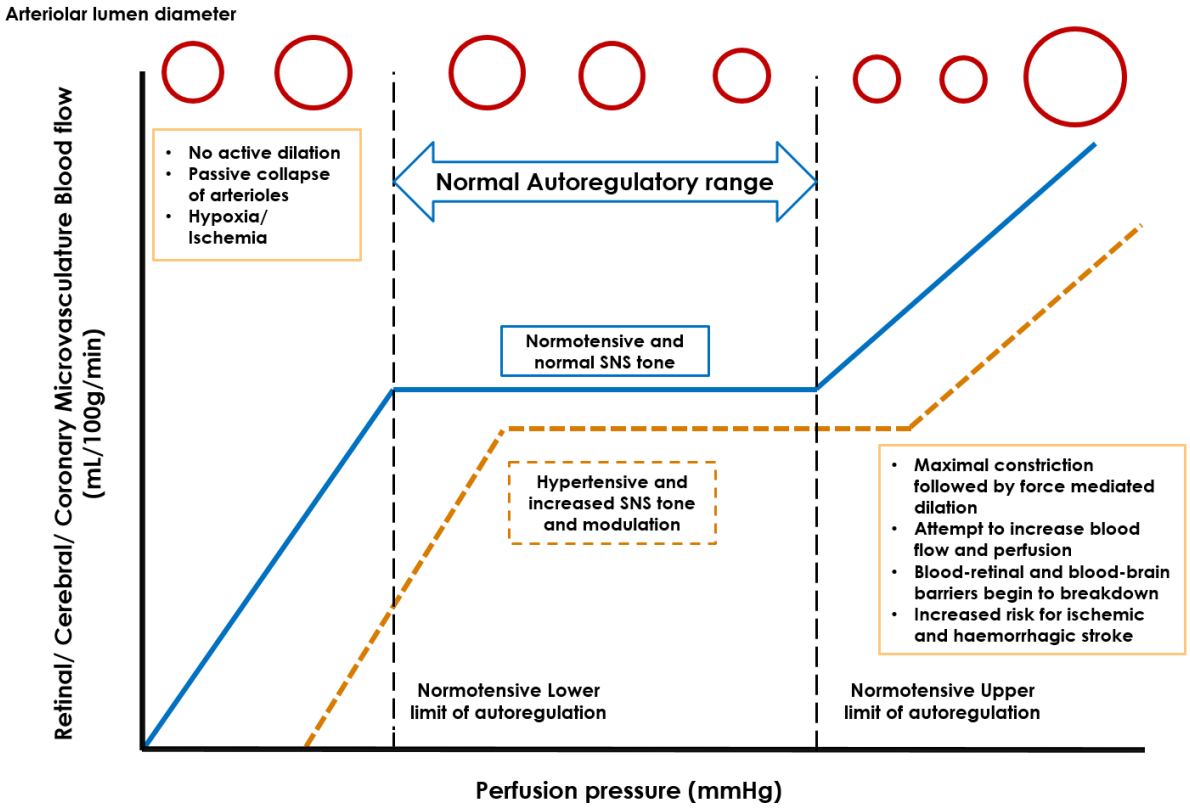

Figure 1.5: The normal autoregulatory capacity and shift that occurs in hypertension. Where: SNS, sympathetic nervous system

To ensure an unbiased approach, aside from being a fundamental physiological process, blood-flow and the regulation thereof highly depend on the ground rules of *Eulerian* and *Langrarian* principles (87). Any microcirculatory system adheres and will adjust to specific changes (such as flow speed, diameter and particle saturation) that occur elsewhere in the arterial tree. These alterations in hydrodynamics, also pertaining to rheology, occur due to the general governing laws of fluid motion, namely conservation of mass, momentum and energy. Keeping with the DVA methodology where FLIP changes in an arteriole or venule are assessed at a specific section, we can apply the *Eulerian* description (also known as the control volume approach) to describe flow at a fixed point in a system – in this case microvasculature – as a function of time (87). Thus these basic principles are sufficient to describe pressure, flow and volume control in the microvasculature. In accordance with these principles any changes (brought about by e.g. SNS stimuli, neural drive and cardiac stress etc.) that occur prior to a fixed point (microvasculature) in the hydrodynamic system, will directly affect the dimensions, volume and flow at this fixed point – ergo, the laws pertaining to conservation. If, hypothetically, there were to be disruption or denervation of these physiological signals (such as increased SNS activity, disrupted neural output, increased cardiac stress) controlling the circulation preceding the microvasculature – therefore the forces acting on the control volume, this disruption will be noted at microvascular level due to these laws (**Figure 1.6**). Hence the degree of autoregulation will depend on the degree of change in the systemic regulation prior to the microvascular bed, regardless of forces/factors responsible for these systemic changes. However, the *mathematical modelling* and description of retinal blood-flow regulation is not the main focus of the current thesis.

It is thus justifiable to assume that elements responsible for changes in systemic BP and cardiac loading conditions (such as sustained SNS hyperactivity) will reflect or indicate the condition and competency of the retinal microvasculature regulatory mechanisms (myogenic, neurogenic and metabolic) to such changes. Despite the structural homologies previously discussed, clinical applicability and risk stratification potential, a direct association between retinal vessel structure and function and traditional risk factors, are lacking. Again, such a relationship may elucidate a more defined brain-retina-heart link.

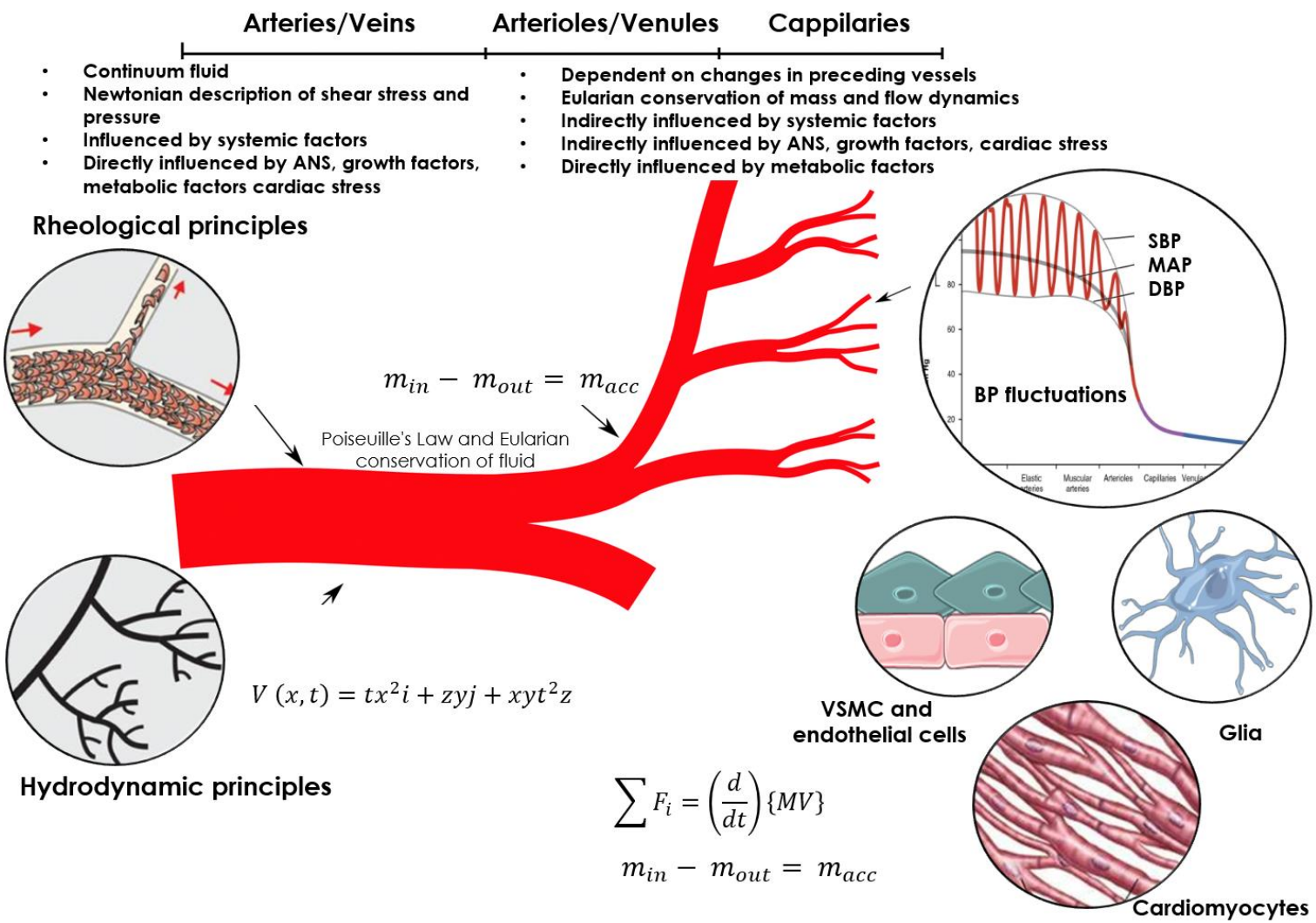

Figure 1.6: Changes that occur in the microvasculature based on changes in the larger arterial tree

Where: ANS, Autonomic nervous system; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, Mean arterial pressure; VSMC, vascular smooth muscle cells; *m*, mass; *acc*, acceleration; *V*, velocity*; F*, force.

5.1.2. Cardiac stress and microvascular structure and function

Altered systemic hemodynamics imply changes in the strain exerted on and pressure created by the cardiac muscle itself. Various pathological conditions have been linked to increased myocardial strain, yet the relationship between cardiac stress and the microvasculature remains under-investigated. This prompts the question: whether traditional, systemic markers of cardiac stress can be linked to microvasculature structure and function and contribute to a combined approach to risk stratification.

Cardiac troponin T (cTnT)

Cardiac troponin T (cTnT) forms part of the cardiac troponin complex. The entire troponin complex itself consists of three sub-units; troponin T, I and C, all of which contribute to force generation during cardiac muscle contraction. cTnT is specifically involved in forming the troponin-tropomyosin complex, as cTnT interlocks troponin and tropomyosin (88) (**Figure 1.7**).

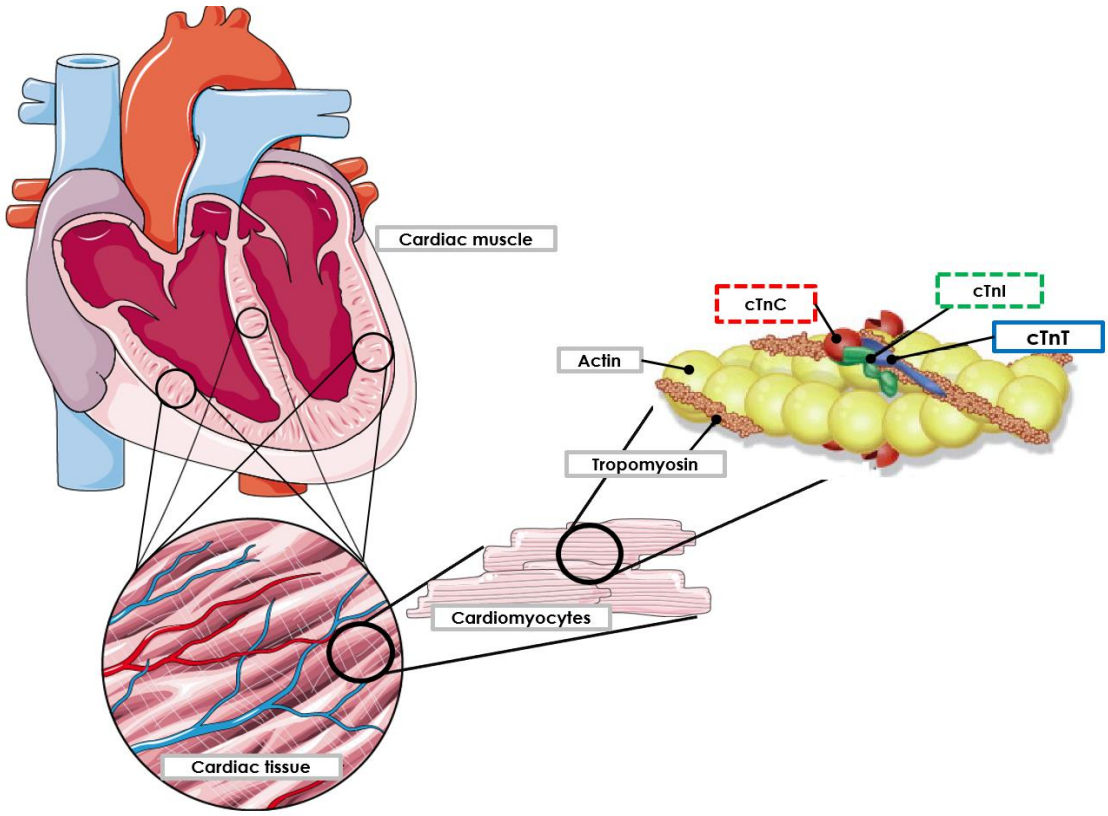

Figure 1.7: A schematic representation of the cardiac troponin T and affiliated structural complexes. Where: cTn, cardiac troponin (T) / (I)/ (C)

In the cytosol, cTnT is found both in free and protein-bound forms. The unbound or free pool of cTnT is the main source of cTnT released in the early stages of myocardial injury (89). The cTnT that is protein-bound is released as the myofibrils degrade, the end result being irreversible myocardial damage. Leakage from reversibly damaged cardiomyocytes, due to increased myocardial strain and excessive volume-loading prior to ischemia may release cTnT (88, 90). Investigations regarding cTnT usually describe its properties as a diagnostic marker and/or therapeutic target (89-91). Yet, cTnT elevation may also be present in several other conditions affecting cardiomyocyte integrity and reflecting microvascular disturbances, such as congestive heart failure, stroke and atrial fibrillation (89). cTnT's release from cardiomyocytes has been linked to an increase in cardiomyocyte wall permeability, myocyte apoptosis and necrosis (88, 89, 92). In a healthy reference population the upper limit for cTnT levels in the circulation is <0.01 ng/mL – usually undetectable by typical analytical procedures (88). cTnT has also been identified as a potential biomarker for CVD risk in the general population (92). This approach has arisen due to the introduction of higher sensitivity troponin assays. Elevated levels of cTnT are defined as a cTnT level exceeding the 99th percentile value of a healthy reference population (91).

Recent population-based studies in which cTnT levels were associated with adverse outcomes include the SABPA study (increased myocardial ischemia and 24H diastolic hypertension risk) (10-12, 72), Dallas Heart study (ischemic heart disease and stroke risk) (89), a study conducted in China (ischemic heart disease and ventricular hypertrophy) (93) and the Atherosclerosis Risk in Communities study (ischemic heart disease) (94). Data on all-cause mortality were provided in 66% of the studies reporting clinical outcomes pertaining to increased cTnT levels' relation to increased cardiovascular-related mortality in these populations studied (94). Reported results demonstrated a significant relationship between cTnT concentration increase (per unit cTnT) and increasing risk of cardiovascular outcomes – establishing cTnT as a continuous variable (88, 89, 95). However, the aforementioned studies extended this result to demonstrate the significance of elevated cTnT in asymptomatic individuals from a general population. In individuals who did not meet diagnostic criteria for myocardial infarction and ischemia, increased high sensitivity cTnT was associated with a greater incidence of myocardial infarction, structural and functional heart diseases (i.e. diastolic dysfunction), cardiovascular mortality and all-cause mortality (89, 92, 96). Hence elevated levels of cTnT can be considered one of the most proximal sentinel markers of heart disease and ischemic damage.

The mechanisms of sub-clinical cTnT elevation in apparently healthy individuals are not fully understood (88). The widespread prevalence of detectable cTnT levels in healthy populations appears to contradict the traditional assumption that cTnT release into the systematic circulation only occurs in the presence of clinically relevant myocardial necrosis (88). There are several possible theories

that might explain this occurrence (72, 88, 96). It is possible that sub-clinical plaque rupture with minimal concurrent necrosis may lead to cTnT release, even in the absence of clinical symptoms. Alternatively, isolated ischemia may be sufficient to facilitate cTnT release from the myocyte cytosol, without the incidence of infarction. Be it the former or latter, either instance involves some kind of myocardial injury, albeit permanent or temporary, causing a cTnT elevation and adverse cardiovascular outcomes in individuals.

A more recent discovery implied elevated levels of cTnT during acute mental stress application, suggesting a relation to the SNS (72). The latter was observed to differ within ethnic groups of the SABPA study. Africans exhibited increased cTnT levels after application of an acute mental stressor. Contrarily, Caucasians exhibited decreased levels of cTnT after acute mental stress application. The SABPA Africans present with a hyperactive SNS (6, 12, 72). It was shown that chronic stress induces SNS hyperactivity, resulting in pressure overload and myocardial ischemic events, exclusively in the Africans in the SABPA study (10-12). In urban-dwelling Africans acute mental stress was associated with hemodynamic α-adrenergic vascular reactivity responses (6). Their α-adrenergic reactivity profile involved increases in diastolic blood pressure (BP), total peripheral resistance, and decreases in stroke volume, cardiac output and arterial compliance (6), which may reflect volume overload. In contrast, their Caucasian counterparts predominantly presented hemodynamic, central cardiac β-adrenergic responses with increases in systolic BP, heart rate, stroke volume, cardiac output, and decreases in total peripheral resistance (6). Additional cTnT release has been linked to acute increased activity of the midcingulate and subgenual anterior cingulate, located in the prefrontal cortex and identified as a primary seat of SNS activity (98). Increases in cTnT accompanied an α-adrenergic response pattern in Africans, and a central cardiac β-adrenergic response accompanied decreased cTnT levels in Caucasians (72). cTnT released in response to SNS activation was linked to catecholamine overload and myocyte necrosis (7).

Indeed, cTnT's relation to specific hemodynamic responses motivates the assumption that cTnT will also be linked to microvasculature and endothelial disturbances (98). cTnT release correlated with diastolic volume-loading and coronary microvascular dysfunction in non-ischemic heart failure patients (99). In support of this finding, small increases in cTnT levels were linked to endothelial dysfunction and small vessel disease (cerebral and coronary), rather than myocardial damage exclusively (98). Higher cTnT levels were identified as an independent stroke-risk marker and predictor of cerebral micro-bleeds (100, 101). No study has ever related cTnT levels to retinal vessel structure or function. Due to cTnT's association with mental stress, microvascular as well as endothelial function, an association between systemic cTnT and possible changes in retinal vasculature responses (during flicker-light-induced stimulation) may elucidate a brain-heart link, specifically when a sympathetic hyperactive status exists.

Amino-terminal pro-B-type natriuretic peptide (NT-proBNP)

Various investigations within the SABPA population have established a positive relationship between cTnT and amino-terminal pro-B type natriuretic peptide (NT-proBNP) (71, 72, 102). B-type natriuretic peptide (BNP) is a ringed peptide secreted by the heart and brain to regulate fluid balance and blood pressure (103). BNP is stored in membrane granules in the ventricles in an inactive, pre-hormone form (proBNP) (103). The proBNP is released in response to ventricular volume expansion and/or pressure overload, and its N-terminal rapidly enzymatically cleaved to obtain biologically active BNP (104). All natriuretic peptides are synthesized as pre-hormones and subsequently cleaved by multiple enzymes to become biologically active (**Figure 1.8**). Physiologically, BNP regulates the water and electrolyte balance ensuring sustained normal blood pressure. It does this by inhibiting the renin-angiotensin-aldosterone system as well as the SNS directly modifying vascular function (104).

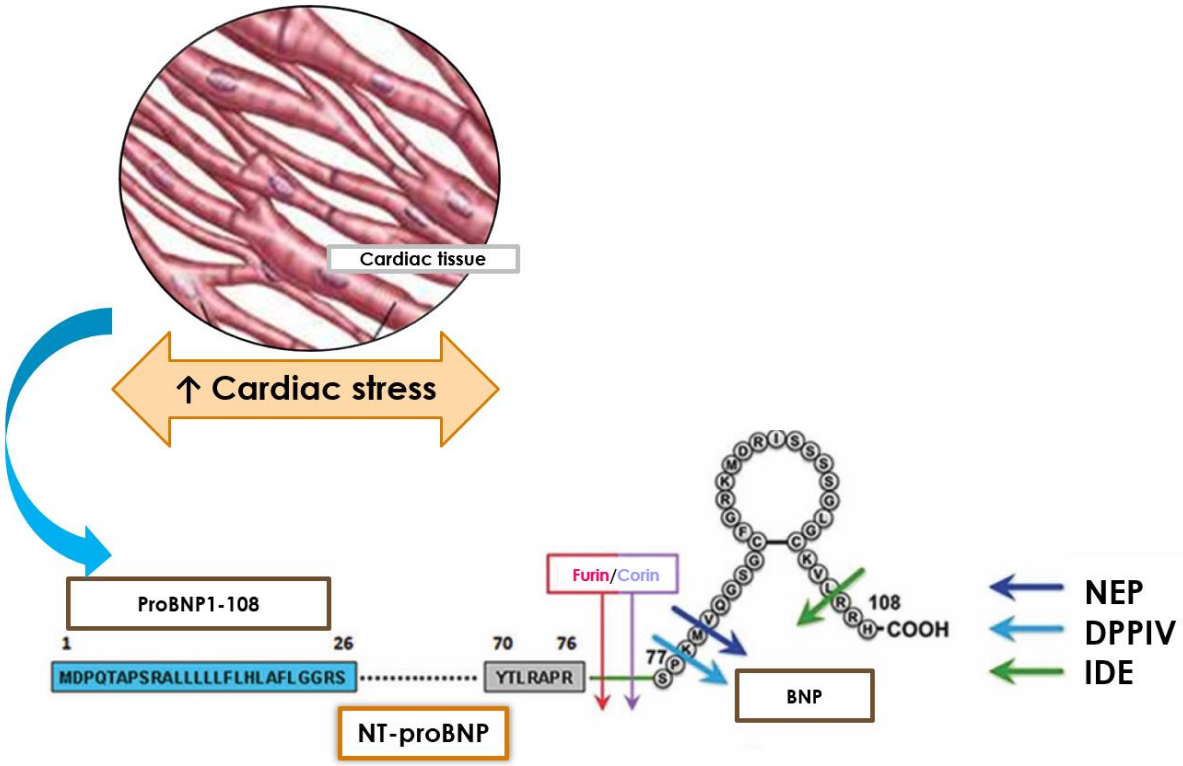

Figure 1.8: Schematic representation of the release and enzymatic activation of NT-proBNP to BNP. Where: NT-proBNP, amino-terminal pro-B-type natriuretic peptide; NEP, nor-endopeptidase; DPPIV, Di-peptidyl peptidase 4; IDE, insulin degrading enzyme

Both BNP and NT-proBNP are markers of ventricular and to a lesser extent atrial distension due to increased intra-cardiac pressure. Circulating levels of BNP have also been closely associated with decreased ventricular contractility, dilation of the left ventricle as well as increased ventricular compliance (104, 105). NT-proBNP is traditionally regarded as a reliable biochemical marker of cardiac conditions such as heart failure, arrhythmias, congestive heart failure and cardiac hypertrophy (105). Reference values for NT-proBNP are highly dependent on age and gender, but on average the normal range for a healthy individual is <100pg/mL (103). Recently NT-proBNP has

also been identified as a robust prognostic marker for the prediction of cardiovascular disease risk in the general population (103, 105). Again, however, NT-proBNP displayed an ethnic-specific risk probability.

Ethnic disparities in NT-proBNP have recently been reported (71, 72, 102). In the SABPA study, Africans exhibited higher baseline NT-proBNP levels and greater increases in NT-proBNP during acute mental stress application, than lower levels and decreases during acute stressor application in Caucasians (72). Here increased NT-proBNP levels indicate an increase in cardiac stress due to SNS hyperactivity and again accompany an α-adrenergic hemodynamic response pattern. Increased cardiac stress may contribute to volume-loading and pressure build-up in the venous system, if sustained.

Longitudinally, however, Africans displayed a downregulation in NT-proBNP levels (102). This may indicate that chronic, sustained increased cardiac stress, pressure overload and hypertension may desensitise tissue to the cardio-protective effects of NT-proBNP. Furthermore, lack of such a cardio-protective effect may cause functional and structural alterations already manifesting at microvascular level.

NT-proBNP and retinal microvasculature

It is clear that NT-proBNP is a recognised dynamic marker of cardiac stress and vascular damage relating to large vessel diseases. However, there are indications that NT-proBNP also relates to small vessel diseases (107, 108). Recent studies showed that NT-proBNP levels were associated with cerebral small-vessel diseases including white matter lesions and silent brain infarcts (107). NT-proBNP is also functionally linked to glial cell regulation (108) and recently, directly with retinal microvascular damage (108). The human retina does possess a well-developed natriuretic peptide system (107). *In vitro* studies have shown that hypoxic conditions stimulate the active release of BNP from human retinal pigment epithelium cells (107). Indeed, systemic levels of NT-proBNP relate closely to those measured directly in the cerebral spinal fluid (109).

Aside from cardiac stress-induced NT-proBNP release, glial cells have been identified as sources and targets of BNP (110). Retinal autoregulation is also controlled by glial cell activity. Indeed, glial cells not only regulate the release of BNP, but certain actions of glial cells are also controlled by the binding of BNP (110). Expression of BNP has also been detected in astroglial cells, which cohesively function with the microvasculature (108, 110). In the rat retina, immunoreactivity for BNP was strong in the astrocytes and trunks of the Müller cells (110). This suggests that BNP may be involved in glia-ganglion communication. Additionally, the end-feet of Müller cells, closely enwrapping retinal blood vessels, also suggest that BNP may participate in the direct regulation of retinal blood flow and maintenance of IOP. Aside from BNP's direct influence on retinal blood vessels (108), it may also exert an indirect effect. *In vitro* studies have shown that BNP activates both the endothelial

and vascular smooth-muscle cells' guanylate cyclase receptors, which promotes arteriolar vasodilation via ion-channel activation (108, 111). Additionally, BNP also stimulates the production of nitric oxide, a potent local vasodilator (112).

Lower levels of NT-proBNP are associated with early microvasculature changes, including loss of endothelial integrity (111), hemodynamic modifications and reduced densities of both coronary and cerebral microvasculature, signifying an increased risk for cerebrovascular events, specifically ischemic stroke risk (113). Contrarily, Mutlu and co-workers (108) found that higher levels of NT-proBNP related to narrower retinal arterioles. This might possibly be explained by way of acute/ chronic functional and structural changes that may occur in the retinal vasculature. It is possible that sustained cardiac stress, as observed in a hypertensive setting, may lead to altered function and remodelling, preceding rarefaction. Such changes will alter local blood supply, increasing resistance and adversely affecting perfusion pressures (**Figure 1.9**). However, long-term volume loading and chronic, sustained cardiac stress may present with a different NT-proBNP profile and risk association. These apparent contradictions warrant further investigation regarding NT-proBNP's role in microvascular structure and function. Additionally, variations in NT-proBNP levels have also been related to alterations in sympathetic activity (105). Indeed, BNP directly interacts with the SNS by decreasing SNS tone, elevating BP, decreasing peripheral resistance and relieving increased cardiac strain (72, 104). Low NT-proBNP levels are associated disrupted autonomic innervation (105). Yet again the influence of the SNS is evident.

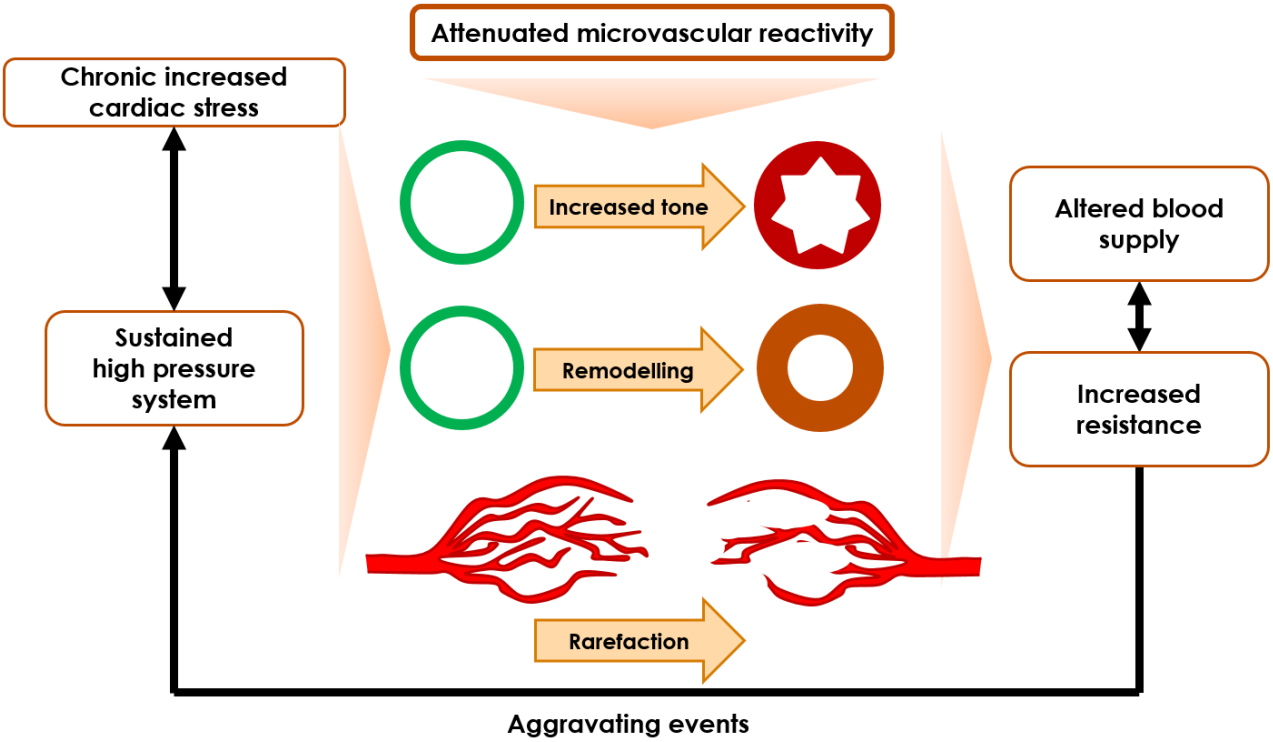

Figure 1.9: Hypothetical series of events where sustained cardiac stress, as observed in a hypertensive setting, may lead to altered function and remodelling, preceding rarefaction, altering local blood supply, increasing resistance and adversely affecting perfusion pressures

5.2. Neurogenic influence on retinal blood-flow

Until recently, it was generally accepted that the autoregulatory mechanisms of the retinal circulation are not subject to neurogenic control. A great deal of controversy exists due to the widely accepted notion that the retinal vasculature is devoid of sympathetic influence, and innervation, beyond the level of the lamina cribrosa (114-116). However, histological evidence suggested the presence of alpha-adrenergic receptors in the mammalian retinal vessels (117-119), and the retinal vessels do contain catecholaminergic amacrine cells.

The choroidal circulation is under undebated neurogenic control and is subject to sympathetic-stimulated vasoconstriction via noradrenergic and neuropeptide-Y fibres (32, 120). Therefore the choroidal circulation, as well as the extra ocular circulation, may further contribute to and influence the degree of myogenic retinal autoregulatory mechanisms. Both infer that the SNS may directly influence the contractile abilities of the retinal microvasculature. Irrespective of these mechanisms, systemic hemodynamics may alter autoregulation capacity, depending on the degree of hemodynamic change that occurs prior to the microvasculature bed (*Eularian conservation of Fluid*).

5.2.1. Sympathetic function and microvasculature structure and function

Our understanding of the SNS has advanced parallel with the rapid expanding field of neuroscience. Indeed, the advancement in sophisticated techniques has contributed to this expansion, yet the development of new ideas has driven it. SNS activity is mediated through modality-specific afferent activity and their associated reflex circuits (121), but also via functionally selective groups of neurons throughout the central nervous system (121).

Pioneering research by Hilton and colleagues (122, 123) showed that cardiovascular changes elicited by the stimulation of specific brain areas, mimicked cardiovascular changes that occurred during physical-stress application (121). These observations created a shift in the dogma that blood pressure was not solely regulated by lower brainstem centres, but that higher cortical structures are also involved (**Figure 1.10**). This may emphasize that central control is exerted by the brain on all vascular systems (8).

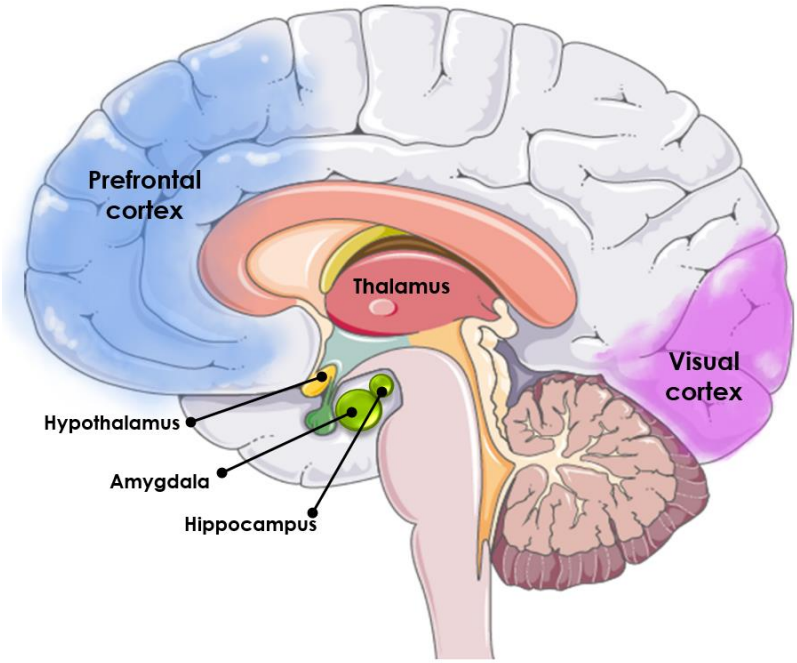

Figure 1.10: The brainstem centres as well as cortical structures involved in and contributing to blood pressure regulation

It has become increasingly clear that any response to stressful stimuli requires precise, differential and selective patterns of SNS activity (3-10, 121). Intrinsic plexuses of neurones and post-ganglionic nerves also supply the smooth-muscle cells and vascular-muscle cells of the blood vessels, mediating such sympathetic actions (124, 125). Such mediating effects are not reserved for larger arteries and veins, but also directly influence the smaller branches of the vascular tree (121).

The SNS and the retinal microvasculature

The influence of the SNS on the retinal microvasculature's autoregulatory capacities has been quite a controversial topic since the early 1950s (32, 115, 116, 125-130). Conflicting reports both confirm and deny the existence of sympathetic innervation of the retinal vasculature.

Using fluorescent histochemical techniques, Laties et al (115) reported that innervation from the SNS to the central retinal artery in the rabbit did not extend past the lamina fibrosa upon entering the eye. However, vasodilation of the rabbit pre-retinal vessels was observed, following a bilateral superior cervical ganglionectomy (129). This suggests that sympathetic nerves were present in the pre-retinal vessels of the rabbit eye. In support of this notion, Matsuyama and co-workers revealed nerve fibres running parallel with the retinal vessels, endorsing the hypothesis of centrifugal nerve fibres in the retina (131). Indeed, adrenergic nerve fibres were observed on the walls of retinal vessels well beyond the lamina fibrosa and for several millimetres proximally on the retinal surface surrounding the optic disc. However, the effects of these receptors, after decades of exploration, are still unknown. Via transmission electron microscopy, scanning electron microscopy and fluorescent histochemical

examination, Furukawa (119) demonstrated an inverse relationship between the number of nerve endings on arterioles and the diameter of the arteriole, with its distance from the optic disc. These nerve endings disappeared following a ganglionectomy, which suggests that sympathetic nerves originating from the superior cervical ganglion innervated the pre-retinal vessels in the rabbit eye (118).

Additional studies indicating the presence of sympathetic binding sites on mammalian retinal vessels also reveal high affinity at alpha-1 and alpha-2 adrenergic binding sites (117). Furthermore, adrenergic receptors can be stimulated by dopamine and vice versa (132, 133). It is possible that these receptors may promote a relaxation response or attenuate a pre-existing contractile response. The exact, cellular mechanism by which intraluminal catecholamines may affect receptors on the vascular smooth-muscle cells are unknown, as the retinal arterioles lack fenestrations and possess tight-junctions in the endothelium (32). Then it is theoretically possible for stimulation to occur when these tight junctions are compromised (i.e. during SNS hyperactivity and the associated increases in intra-ocular pressure, systemic blood pressure, or volume over-load) or when the inner blood-retinal barrier and accompanying ganglion cells are dysfunctional.

A study conducted by Flannigan and co-workers (127) demonstrated that the mean retinal vessel responses to autonomic stimulation were significantly reduced in sympathectomised eyes. These findings suggest that the SNS exerts a significant effect, either directly or indirectly, on the smooth-muscle tone of the retinal vasculature (127). Hence denervation of the SNS may cause loss of tone and/ or altered hemodynamics, and such denervation may occur as an integral feature of a general SNS dysfunction or dysregulation (128). Lanigan's study inevitably implies that the impairment of retinal vascular reflexes is related to the disruption of cervical sympathetic innervation to these vessels. These findings additionally imply that sympathetic reflexes of the retinal vasculature may contribute significantly to the effective autoregulation of retinal blood flow (127).

Malan and colleagues (8) also demonstrated that a chronically challenged SNS (as indicated by a β-adrenergic hyporesponsive profile), was associated with retinal vascular remodelling, specifically in the SABPA study's African men cohort. The observed β-adrenergic hyporesponsivity, due to chronic stress exposure, emphasised the importance of central control by the brain on the circulatory system, regardless of the vascular bed (8).

However, whether SNS alterations influence retinal vessel responses during FLIP, is yet to be determined. Nowhere is the ability of the SNS to contribute to rapid adjustments during situational demands more evident than in the influence on heart rate.

Heart-Rate-Variability (HRV)

A healthy biological system displays complex patterns of variability and situational modifications. As Shaffer & Ginsberg (134) declare: a healthy heart is not a metronome. Constant, complex oscillations allow the cardiovascular system to rapidly adjust to sudden physiological and psychological events that threaten homeostasis. Heart-Rate-Variability (HRV) is the most often employed, non-invasive assessment of autonomic tone and modulation. HRV is the variation in the time interval between successive heart beats (16, 135).

HRV indicates neuro-cardiac function and is generated as a result of brain-heart interactions and dynamic, non-linear ANS processes (**Figure 1.11**).

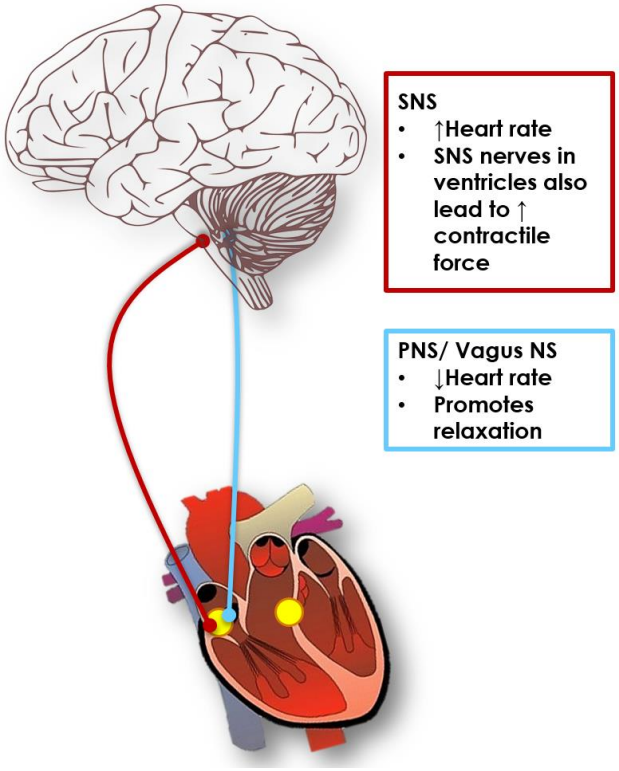

Figure 1.11: The autonomic nervous system control of heart rate via sympathetic and parasympathetic input. Where: PNS, parasympathetic nervous system; SNS, sympathetic nervous system; ↑ increase; ↓ decrease

It is also important to note that most components of HRV provide a measurement of the degree of autonomic *modulation* rather than exacting the level of autonomic *tone* (135). HRV reflects regulation of autonomic balance, BP, heart, but also vascular tone, which refers to the diameter of blood vessels that locally regulate BP and perfusion pressures. Healthy biological systems display a degree of spatial and temporal variation, and such variation is either lost or increased during certain disease states (134). Higher HRV therefore is not necessarily a sign of a healthy condition. Certain pathological conduction abnormalities, such as atrial fibrillation, are accompanied by higher HRV values (136). Optimal HRV is associated with healthy self-regulatory capacity, adaptability and

resilience (134, 135). Vagal-influenced HRV has been associated with greater performance of executive functions such as emotional processing and attention management by the prefrontal cortex (137). Indeed, information processing by the intrinsic cardiac nervous system can modulate fronto-cortical activity (122, 123) and greatly impact on higher-regulatory functions (135).

HRV parameters

HRV can be measured using time domain, frequency domain and non-linear measures (**Figure 1.12**). These measurements can be applied to varying time scales. However, since longer recordings produce more epochs (variations in HR), long-term recordings better reflect processes that have slower fluctuations, those subject to circadian rhythms, for instance (134). The cardiovascular system's reaction to environmental stimuli or changing workloads also differs based on long- or short-term exposure. Short-term and long-term HRV recordings are therefore not interchangeable.

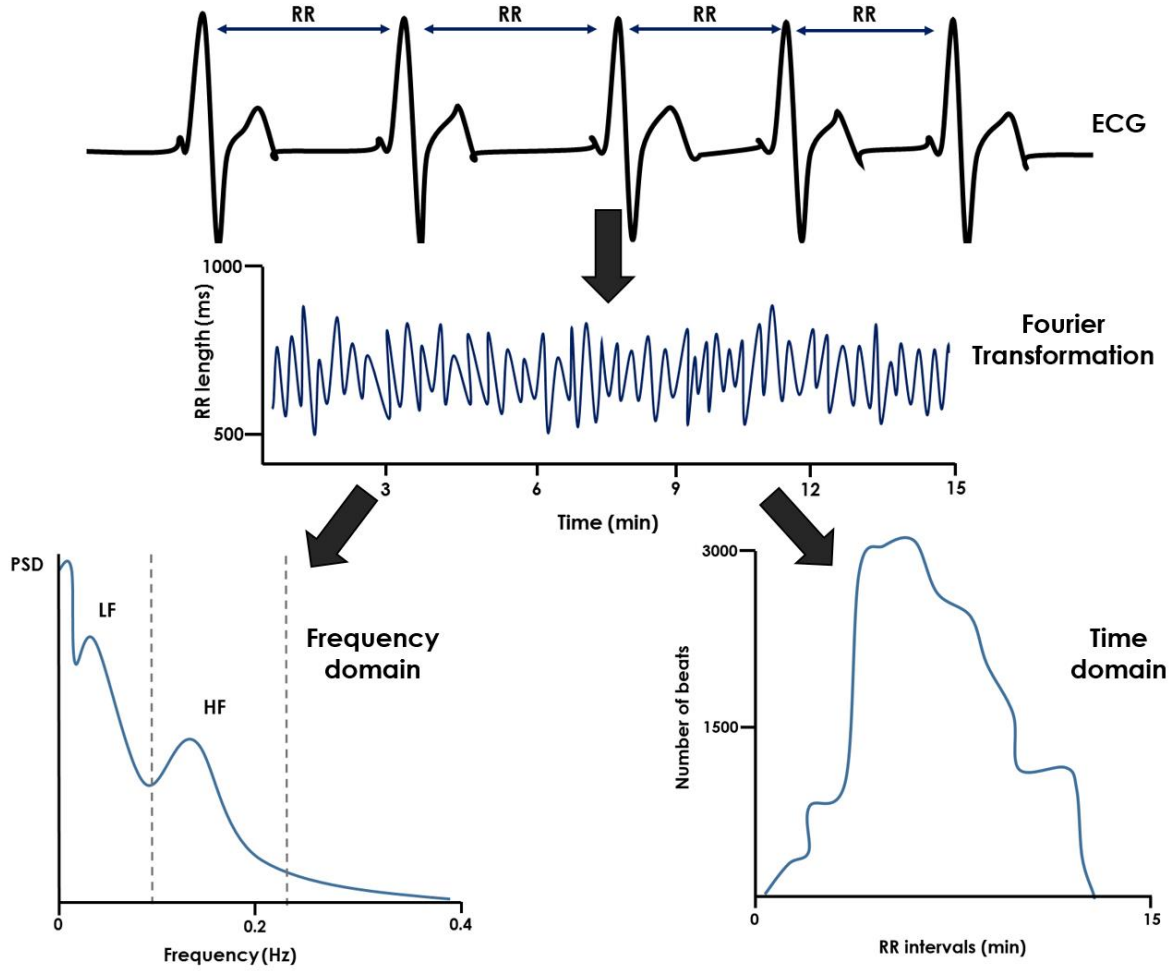

Figure 1.12: Derivation of heart rate variability (HRV) parameters (time and frequency domains) from the electrocardiogram (ECG). Where: PSD, power spectral density

Time domain parameters

Time domain indices of HRV either directly measure the HR at any point in time or the intervals (inter-beat intervals) that exist between normal successive complexes (135). These may be calculated from 24h ECG recordings or may be determined using smaller, isolated segments of the recording.

A frequently calculated HRV variable is the **standard deviation of the NN (inter beat intervals of normal sinus rhythms) intervals** (**SDNN**), also known as the square root of variance (135, 138). Here, the term "normal" refers to the removal of all ectopic beats – those beats that originate outside of the heart's sinoatrial node – prior to calculation (134). Although both SNS and PNS activity contribute to the SDNN, the low frequency bands (SNS indicators) show greater correlation with SDNN (134, 135), specifically during 24h recordings. Therefore, irrespective of whether the SDNN indicates pure SNS or mixed SNS/PNS input, low SDNN values are generally interpreted as increased SNS *activity* (135). Recordings of longer duration are useful in indicating cardiac reactions to a wide range of environmental stimulation. This not only involves cardiac changes due to circadian rhythms, but also the heart's response to anticipatory central nervous system activity (135) and the resultant changes in cardiac workload and cardiac strain. Clinically, the SDNN is regarded as the gold-standard for medical risk stratification, especially when recorded over a 24H period (135-138). Lower SDNN values predict increased cardiovascular morbidity and mortality. Patients with an SDNN value below 50ms are classified as at-risk, 50-100ms indicate a compromised condition and greater than 100ms is indicative of health (135). In infarction survivors, 24H SDNN values indicating greater health had a greater life expectancy than those with lower SDNN values (134-138).

It has been reported within an epidemiological setting that the SABPA African cohort exhibits lower SDNN values, indicative of a sympathetic hyperactive status (10, 72). Lower SDNN values are also associated with an increased risk for myocardial ischemia, stroke as well as post-stroke complications (e.g. reduced re-perfusion and ischemia) (10, 139). Indeed, reduced SDNN values accompany impaired baroreceptor sensitivity, a relationship that is associated with stroke severity (140). Within the SABPA African male cohort, Uys and co-workers showed that attenuated baroreceptor sensitivity indicated autonomic dysregulation, increased myocardial ischemia and myocardial hypertrophic modifications (141, 142). This may further support SNS hyperactivity's contribution to stroke and CVD risk within this population. However, SDNN values measured during short recordings may also be appropriate to determine risk, specifically during taxing or challenging stimuli (135).

The most frequently applied measure derived from interval differences is the **root mean square of successive differences between normal heart beats** (**rMSSD**). The rMSSD is ideal for measuring short-term changes in HRV (135), as it reflects the beat-to-beat variance and is the primary time domain measure used to estimate vagally modulated changes in HRV. Therefore, the rMSSD is regarded as a pure indicator of vagal tone (135-147). Lower rMSSD values are related to an increased

risk score for sudden cardiac death, specifically in epilepsy patients (135). Schuster and co-workers were the first to find that narrower retinal arterioles correlated positively with a decreased 24H rMSSD value (114). This finding indicates that vagal influences may impact on retinal vessels. However, an investigation into HRV parameters measured during a direct challenge on the retinal vessels is yet to be done.

The NN intervals can also be converted and displayed as geometric patterns, such as the **triangular index** of HRV (**HRVti**). The HRVti is an index of the pulse variability based on a triangular interpolation method in the given time interval where cardiovascular risk 0-15 is high; 15-20 is mid; >20 is low (134-139). The histogram assesses the relationship between the total number of RR intervals detected and the RR interval variation. The triangular HRV index is an estimate of the overall HRV (134). Again, in the SABPA African cohort exclusively, a lower 24H HRVti was associated with increased myocardial ischemia and cardiac stress (10). Reduced HRVti also associated with an α-adrenergic response and increased cTnT levels in response to acute mental stress application in Africans (72). The latter further exemplifies a SNS hyperactive status in the SABPA African cohort. None of these associations were evident in the SABPA Caucasian group. Nevertheless, findings regarding ethnic disparities in HRVti are sparse or lacking all-together.

These time domain variables derived from the NN intervals and their clinical applications are summarized in **Table 1.1**.

Frequency domain parameters

Frequency domain methods can be compared with a prism that refracts white light into its component wavelengths (**Figure 1.13**). Applying Fast Fourier transformation (analogue to that of an electroencephalogram) or autoregressive modelling, we can separate HRV components into its different operational frequency bands (134, 138). These bands typically range from high-frequency (HF) (0.15-1.04 Hz) (138), low-frequency (LF) (0.04-0.15 Hz) and the ultra- and very low-frequency domains (≤0.003 Hz – 0.04 Hz).

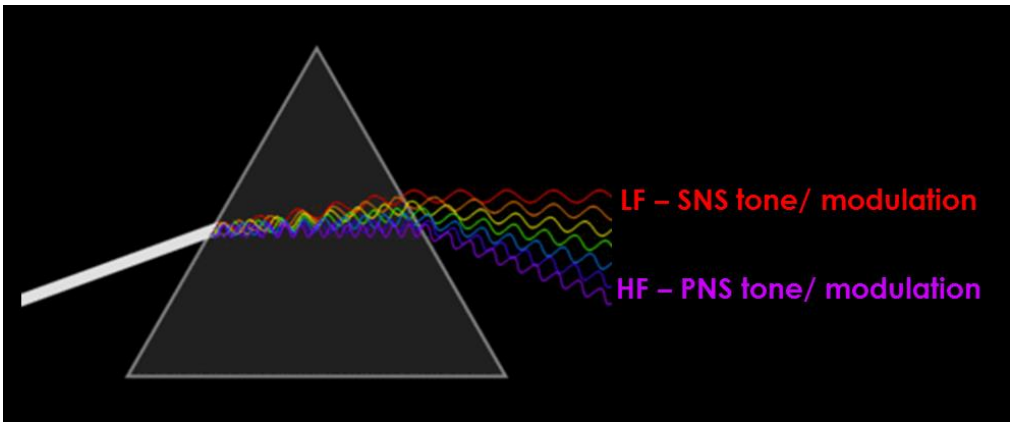

Figure 1.13: Visual comparison of HRV frequency parameters to white light being refracted into component wavelengths. Where: LF, low-frequency; HF, high-frequency; SNS, sympathetic nervous system; PNS, parasympathetic nervous system

Both parametric and non-parametric methods exist, all providing information regarding the power distribution across the different frequencies.

The **HF band** is conventionally recorded over a minimum of 1 minute. It is widely accepted that the HF band is mainly reflective of parasympathetic *modulation*, yet may not be a pure index of cardiac vagal *activity or tone* (135, 138). Lower HF power is associated with mental stress, anxiety and panic (10, 72). Modulation of vagal tone contributes to the dynamic autonomic regulation essential for cardiovascular health. Deficient vagal modulation is linked to increased morbidity, coronary syndromes as well as stroke risk (135, 139). Thayer and colleagues found that lower HF was evident in African American youth than in European-American youth (143). Interestingly, Allen and colleagues found, via functional magnetic resonance imaging investigations, that perfusion to the specific brain regions related to vagal activity related to resting levels of HF power. However, these relationships again differed between European-Americans and African Americans (97). They speculated that the regions of the cingulate and medial prefrontal cortex tonically inhibit sympathoexcitatory neurons in the rostral ventrolateral medulla and excite the vagal efferent in the nucleus ambiguous (97). This suggests that their findings indicate that African Americans with lower resting cardiac vagal activity may also have greater resting perfusion in brain regions associated with regulation of sympathetic activity (97).

The **LF bands** are typically recorded over a minimum period of 2 minutes, ensuring enough epochs within that timeframe (134-138). Traditionally, variations in the LF bands signify SNS modulation, or modulation of SNS tone (135). However, recent evidence suggests that the PNS may also influence the higher frequency values within this band, as the SNS rarely produces rhythms above 0.1 Hz (135). Therefore, when reporting any HRV parameter, and specifically LF, it is essential for both HF and LF domains to be taken into account and it is unconventional to report one without the other. The HF

and LF domains may also be expressed as normalized units (HFnu and LFnu) (138). These units represent the relative value of each power component in proportion to the difference between the total power and VLF component (138). This normalized expression emphasizes the balanced and controlled behaviour of both the SNS and PNS. Additionally, such normalization minimizes the effect of fluctuations in the total power on the individual HF and LF components. Indeed, this makes it essential to report the normalized values specifically during short-term recordings (20 minutes and less).

These frequency domain variables, along with their clinical implication, are summarized in **Table 1.2**.

Table 1.1: Summary of HRV Time-domain parameters used in this thesis

Parameter	Unit	Description	Approximate Frequency domain equivalent	Physiological relevance / Clinical implication
SDNN	ms	Standard deviation of the NN intervals (can be applied to short-term recordings)	Total Power and LF	Depressed SDNN indicates increased SNS activity, evident in hypertensives vs. normotensives (1,2) Depressed SDNN values were observed in acutely stressed Africans than those of Caucasians (2) Lower SDNN values also indicate an increased stroke risk (1, 3-4)
rMSSD	ms	The square root of the mean of the sum of the squares of differences between adjacent NN intervals (can be applied to short-term recordings)	HF	Depressed values indicate vagal withdrawal/ decreased vagal modulation/ tone Hypertrophic cardiomyopathy patients present with depressed values and low vagal tone Low rMSSD values associated with a higher stroke risk (3, 5)
HRVti		Total number of all NN intervals divided by the height of the histogram of all NN intervals. Also defined as a geometric mean derived from time domain parameters (can be applied to short-term recordings, if the window selected provides enough variance).	Total power	Depressed SDNN, contributing to a decreased HRVti indicates a SNS hyperactive state HRVti Evident in hypertensives vs. normotensives (1-3)

Table 1.2: Summary of HRV Frequency-domain parameters used in this thesis

Parameter	Unit	Description	Physiological relevance/ Clinical implication
LF	ms^2	Power in low-frequency range (can be reliably calculated from short-term and 24H recordings)	Lower values indicate greater risk for acute cardiac events (i.e. infarction), congestive heart failure and stroke (1, 5) Less power recorded in hypertensives vs. non-hypertensives

			Post-stroke patients with left-sided stroke showed greater vagal dominance (3-7)
LFnu	nu	Low-frequency power in normalized units LF/(total power−VLF)×100	Lower values indicate greater risk for acute cardiac events (i.e. infarction), congestive heart failure and stroke Less power recorded in hypertensives vs. non-hypertensives Post-stroke patients with right-sided stroke showed greater sympathetic dominance, however overall lower LF values (3, 7)
HF	ms^2	Power in high frequency range (can be reliably calculated from short-term and 24H recordings)	Reduced vagal activity in hypertensives and patients suffering from chronic heart failure (1-3) Lower HF power related to increased stroke risk and poorer post-stroke outcomes Post-stroke patients with left-sided stroke showed greater vagal dominance, however overall lower HF values (1, 3-7)
HFnu	nu	High-frequency power in normalized units HF/(total power−VLF)×100	Reduced vagal activity in hypertensives and patients suffering from chronic heart failure Lower HF power related to increased stroke risk and poorer post-stroke outcomes (4, 5)
LF/HF		Ratio LF [ms^2]/HF[ms^2] (can only be reliably calculated from short-term recordings)	Increased LF/HF was evident in hypertensive and diabetic patients Increased LF/HF was also linked to an increased risk for ischemic stroke (5, 6, 7)

Compiled from:

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The SNS hyperactivity and possible ophthalmological implications

Chemical stimulation of autonomic regulatory neurons in the dorsomedial and perifornical nuclei in the hypothalamus (the seat of SNS activity) showed marked increases in IOP (144). Agnifili et al (145) showed that IOP variation was greater in patients suffering from primary open-angle glaucoma than in normal controls. Such fluctuations in IOP, together with altered hemodynamics and retinal blood-flow regulation, may possibly contribute to the pathogenesis of and glaucomatous damage in primary open-angle glaucoma (144). Indeed, defective perfusion played a role in the appearance and exacerbation of glaucomatous optic neuropathy (142-144). Sympathetic dominance and depressed HRV was also documented in patients with normal-tension glaucoma (144). Additionally, associated vascular dysregulation was linked to SNS hyperactivity, manifested as blunted BP responses and decreased optic nerve head blood flow in response to cold provocation (147). However, whether changes in HRV parameters during a stressor will associate with retinal vasculature responses, is yet to be investigated.

Neurogenic control: Neurovascular coupling

One of the most studied aspects of this neurovascular-unit is its link between neural activity and blood flow. Blood flow proportionally relates to the metabolic demand set upon that specific area (e.g. specific brain region or retina) (31); therefore during conditions that require greater energy consumption such as during application of a stressor, blood flow to that particular area/ areas will increase. However, pre-existing conditions may influence such blood-flow regulation.

Due to their close spatial association with synapses and the microvasculature, astrocytes and glial cells are ideally positioned to link neural activity to microvascular function via a broad range of intra- and inter-cellular pathways (31). The role of astrocytes and glial cells in neurovascular coupling may be dual in nature, influencing the capillaries as well as the arterioles of the retina (148). This supports the hypothesis that astrocytic signals may be critical in linking neural activity and microvascular responses. Additionally, due to arterioles' and capillaries' unique position enabling them to react to neuronal and astro-glial signals, neurovascular coupling can be initiated at microvascular level and be conducted upstream (31, 148).

Endothelial cells respond to and secrete powerful vasoactive agents such as NO, endothelin and prostanoids (149), but their role in neurovascular coupling has only recently received attention. Evidence suggests that the endothelium plays a crucial role in the retrograde propagation of activity-induced neurovascular signals (31, 150). In the retinal microvasculature the partial and temporal correspondence between neural activity and the associated hemodynamic response are not precise (31). Local increases in blood flow may exceed the area of activation, affecting downstream arterioles'/ venules' diameters and tissue perfusion. This is defined as retrograde propagation of

vascular response. Again, this corroborates with the basic principles of hemodynamics, that in a vascular network, there will be a coordinated dilation of down-stream and upstream vessels to increase flow whilst avoiding "flow steal" from interconnected vascular areas (150). Therefore the retinal arterioles will be an important site for the control of flow, and changes induced by neural signals will have to be conveyed to the upstream arterioles and capillaries, further down from the area of activation, to ensure flow efficiency. Indeed, this principle is well documented in cerebral pial arteries and arterioles, where vascular responses generated near the area of activation are conducted in a retrograde fashion along the blood vessels engaging pial arteries downstream (31, 149) (**Figure 1.14**). In retinal and systemic vessels, the endothelium also participates in the retrograde propagation of vascular signals (150). This propagation may also influence vasomotor responses, both vasodilation and vasoconstriction, by 1) directly influencing the SMCs and pericytes; and/or 2) the agents contributing to such vasomotor action such as acetylcholine, the catecholamines and possibly traditional growth factors such as BDNF (31).

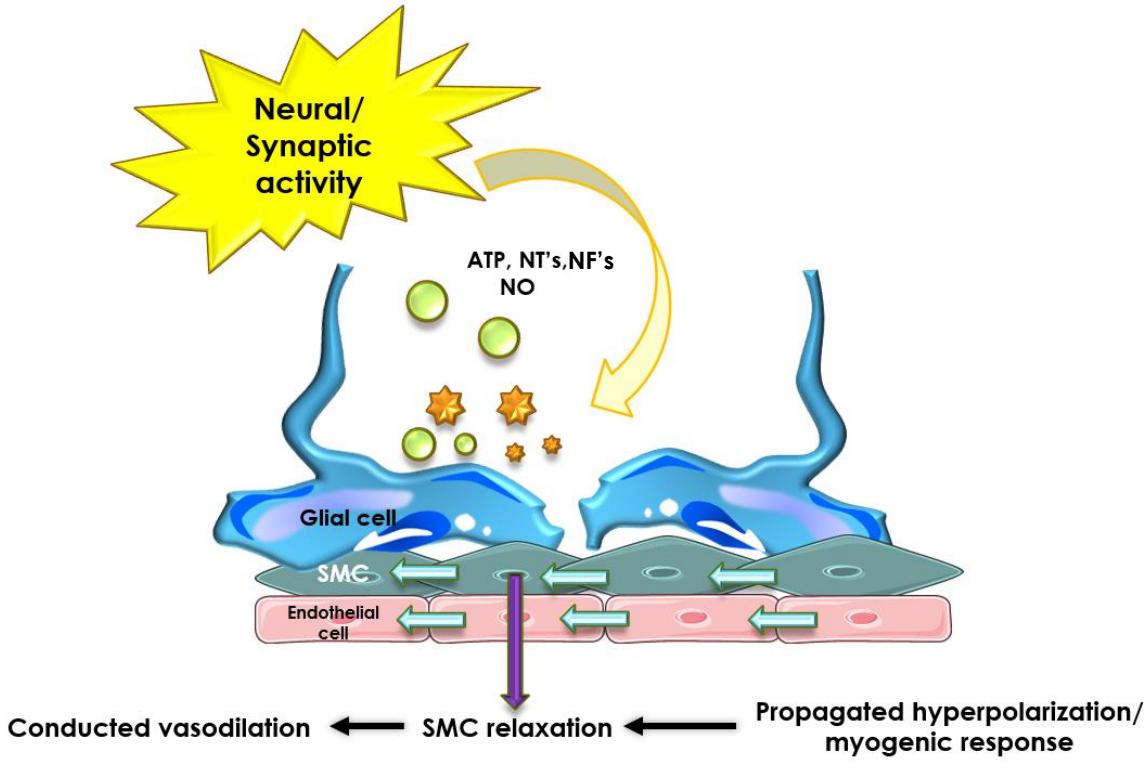

Figure 1.14: Graphical representation of a vascular response generated near the area of activation conducted in a retrograde fashion along the blood vessels, which will engage the arterioles downstream. Where: ATP, adenosine triphosphate, NT's, neurotransmitters; NF's, neurotrophic factors; NO, nitric oxide; SMC, smooth muscle cell.

5.2.2 Brain-derived Neurotrophic Factor (BDNF)

Recent evidence suggests that Brain-derived neurotrophic factor (BDNF) may modulate regulatory pathways (151). Indeed, BDNF is known to modulate the angiotensin signalling in the mammalian hypothalamus in order to increase blood pressure (152). On the contrary, increased expression of BDNF in the paraventricular nucleus of the hypothalamus, to facilitate increases in blood pressure, heart rate and sympathetic tone in response to acute stressors in animal models, has been reported (151). BDNF's modulating abilities will directly be altered by a sustained sympathetic hyperactive state, possibly reflected by decreased levels (153). Higher systemic BDNF levels have indeed been linked to increased activity in the cingulate and medial prefrontal cortex – areas associated with SNS activity (132). Yet it is possible that a sustained SNS hyperactive state may lead to blunted or diminished BDNF action, resulting in lower BDNF levels over-time.

BDNF is a small, secreted protein that forms part of the greater neurotrophin family, which includes nerve growth factor, neurotrophin 3, 4, 5 and 6 (50). BDNF's synthesis occurs in the endoplasmic reticulum as a precursor protein (proBDNF) that moves through the trans-Golgi network. In the presence of lipid-raft sorting receptor carboxy-peptidase E, proBDNF is sorted into vesicles (released in an activity-dependent manner). The terminal of proBDNF is cleaved by protein convertase to finally yield biologically active, mature BDNF (151, 153-159). Biologically active BDNF binds to its high-affinity receptor, tropomyosin receptor kinase B (TrkB), present on both the pre-and post-synaptic terminals (48, 160, 161) of neurons, astrocytes, endothelial and smooth-muscle cells (**Figure 1.15**). It is utterly important to note that BDNF levels measure in serum samples, correlate with those found in plasma, cerebrospinal fluid as well as grey and white matter (162). Thus systemic levels reflect the central level of BDNF.

BDNF is traditionally known to play a key role in synaptic communication, plasticity, neuronal differentiation, maturation and survival (40, 151). In addition, recent evidence suggests that BDNF is produced and released in a neuronal activity-dependent manner, provoking short- and long-term changes in synaptic function (40). Aside from BDNF's direct influence on central control mechanisms responsible for blood pressure modulation, BDNF regulates the expression and maintenance of SNS nerves (163). It has also been speculated that BDNF may contribute to sympathetic hyperactivity transformation, evidenced by decreased BDNF levels (164).

BDNF additionally plays a central role in energy homeostasis, and has a pronounced anti-diabetic effect (165). Both BDNF and its affiliated receptor TrkB are highly released and expressed in several hypothalamic and hippocampi nuclei, known to be involved in glucose and energy homeostasis, but also relate to SNS activity (165). Tonra et al (166) showed that systemic administration of BDNF decreased fasting blood glucose levels in non-insulin-dependent diabetic mice.

Intracerebroventricular administration of BDNF lowered blood glucose levels in a dose-dependent manner in insulin-dependent diabetic mice. Human studies support BDNF's role in energy homeostasis (151, 165). Decreased BDNF levels were observed in women with low insulin sensitivity, and BDNF positively associated with insulin resistance (HOMA-IR) in a cohort of healthy men (167). Indeed, SNS hyperactivity is frequently reported in diabetic patients (76, 167). These results suggest that BDNF may ameliorate systemic glucose balance and alter insulin sensitivity, which may contribute to and possibly be influenced by SNS hyperactivity.

The influence of BDNF on cardiac stress, blood pressure regulation and glucose metabolism denotes BDNF's role in cardiometabolic diseases. Within the SABPA population, lower BDNF levels indeed support the increased cardiometabolic risk profile observed in the SNS hyperactive African group (153). Recent findings within the SABPA study also indicate that lower BDNF levels possibly contribute to disturbed structural endothelial function. This emphasizes the crucial central neural regulatory role of BDNF, specifically considering cardiometabolic dynamics.

From the fore-going discussions it becomes clear that BDNF is uniquely capable of exerting neuroprotective properties, specifically during circumstances of increased metabolic demands such as cerebral ischemia, hypoglycaemia and neurotoxicity induced by glutamatergic activation (132, 168). Recently BDNF has also been identified as an essential element in neurovascular coupling (132, 169, 170). It has been proposed that BDNF may exert a prominent, direct neuroprotective effect in processes that may lead to retinal cell death (170).

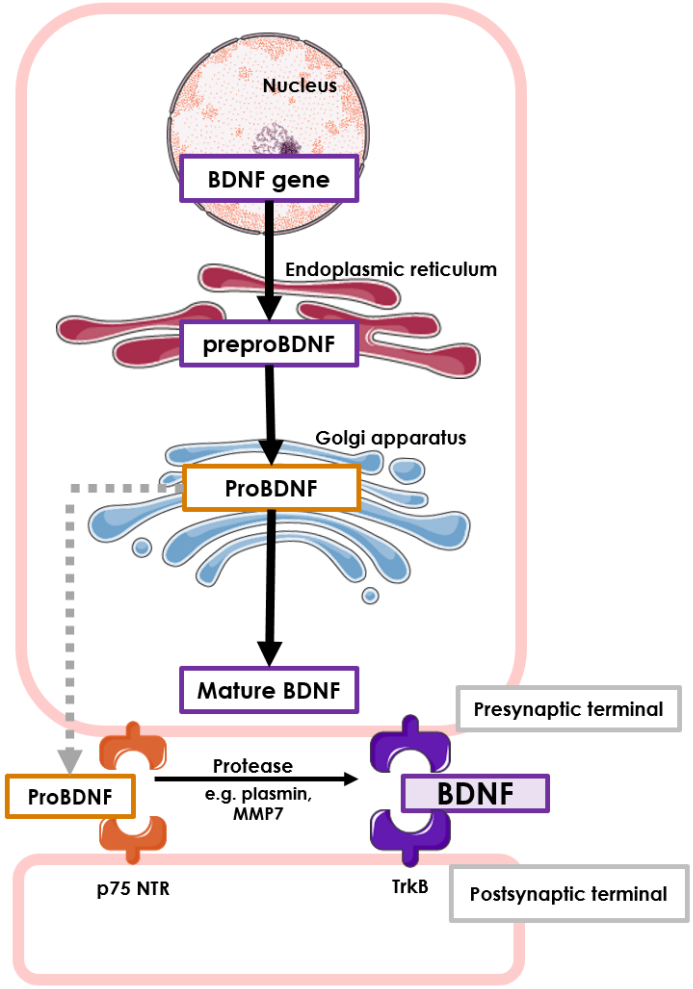

Figure 1.15a: BNDF synthesis, release and binding. Where: BDNF, brain-derived neurotrophic factor; MMP, matrix metallo-proteinase.

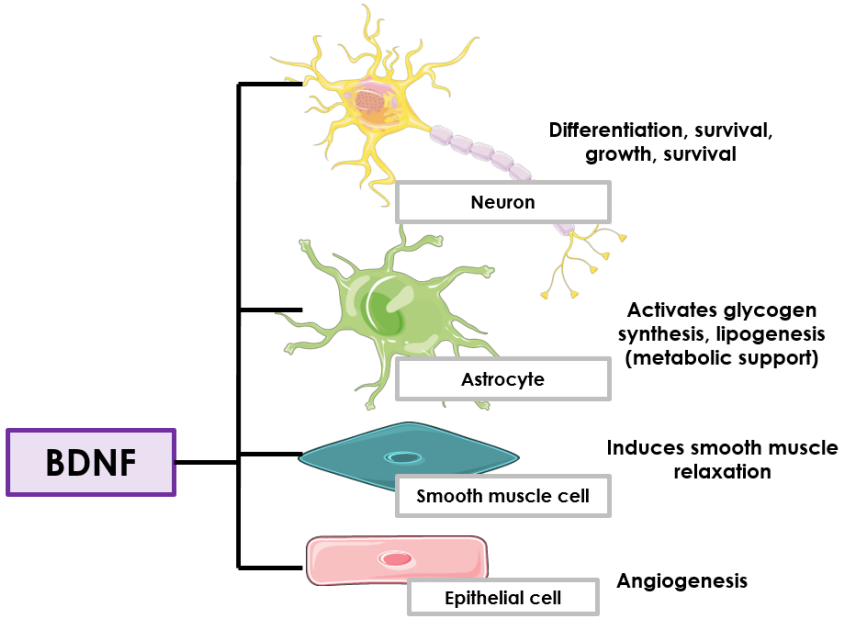

Figure 1.15b: Biologically active BDNF's effects neurons, astrocytes, endothelial and smooth muscle cells. Where: BDNF, brain-derived neurotrophic factor.

BDNF and the retinal microvasculature

Of all neurotrophins, BDNF and its TrkB receptor are the most abundant in the retina (171, 172). BDNF is regulated, in part, by neuronal activity-dependent mechanisms (50, 172), possibly constituting a link between maintaining retinal blood flow during conditions of increased metabolic demand – such as during FLIP. Indeed, recent investigations have targeted BDNF as a possible therapeutic agent in preventing, treating and combatting some key neurological conditions including stroke (170, 173), traumatic brain injury (174, 175), depression and anxiety (50, 132). A large body of evidence supports the key role of BDNF in proliferation, development, differentiation and survival of retinal cells (170, 176). Findings suggest that compromised BDNF levels in the superior colliculus may contribute to the apoptosis of retinal ganglion cells (171, 172). Retinal ganglion cells, retinal pigment epithelial and Müller glial cells all express the TrkB BDNF receptor (171). The presence of these BDNF receptors on these cells does not only promote neurite growth, but possibly also counteracts retinal degeneration and damage (171, 172).

Indeed, low serum BDNF levels are linked to retinopathy (172, 177), increased ischemic stroke risk (178), depression (50), stress, anxiety and SNS hyperactivity (153,179). Regarding glaucoma, evidence demonstrated a substantial increase in neurotoxic materials as well as down-regulated expression of BDNF and the TrkB receptor in the optic nerve tissue (172) and serum BDNF (176). Additionally, increased systemic blood pressure that leads to the impaired retrograde transport of BDNF in the development of glaucoma is proposed to be a key mechanistic feature behind optic nerve atrophy and ganglion cell death (172). The effect of low serum BDNF levels on the retinal vessel dynamics, during FLIP, specifically in human subjects, is yet to be investigated.

6. Problem statement and Motivation for this SABPA affiliated study

This study is important as it will contribute to establishing a potential mechanistic model of the brain-retina-heart link. As the retina is the only location where the microvasculature can be viewed non-invasively, the proposed brain-retina-heart link may be an essential mechanistic pathway within the context of CVD, myocardial ischemia and stroke. The global burden of CVD and incidence of myocardial infarction and ischemic stroke has globally increased, specifically in individuals of African descent. Assessing the brain-retina-heart link may thus expand translation knowledge. Additionally, recent findings within the SABPA population have indicated that Africans persistently exhibit a SNS hyperactive status, accompanied by hypertension, myocardial ischemia and stroke risk. However, to establish a brain-retina-heart link, a step-by-step process needs to be followed. These observations therefore served as motivation to research the relationship of novel SNS-associated cardiac stress markers, cTnT and NT-proBNP and the retinal microvascular structure and function –

investigating the retina-heart link and associated retinal myogenic control mechanisms. Following this motivation, investigating the controversial topic of SNS influence (HRV) on the retinal microvasculature's structure and function becomes crucial – to establish a brain-retina-heart link and explore the neurogenic control of the retinal vasculature. Finally, these approaches prompt research regarding the influence of the most abundant neurotrophic factor in the retina (BDNF), which is linked to both cardiac stress and SNS hyperactivity, on retinal vascular dynamics – exploring the role of neurovascular coupling on retinal vascular dynamics.

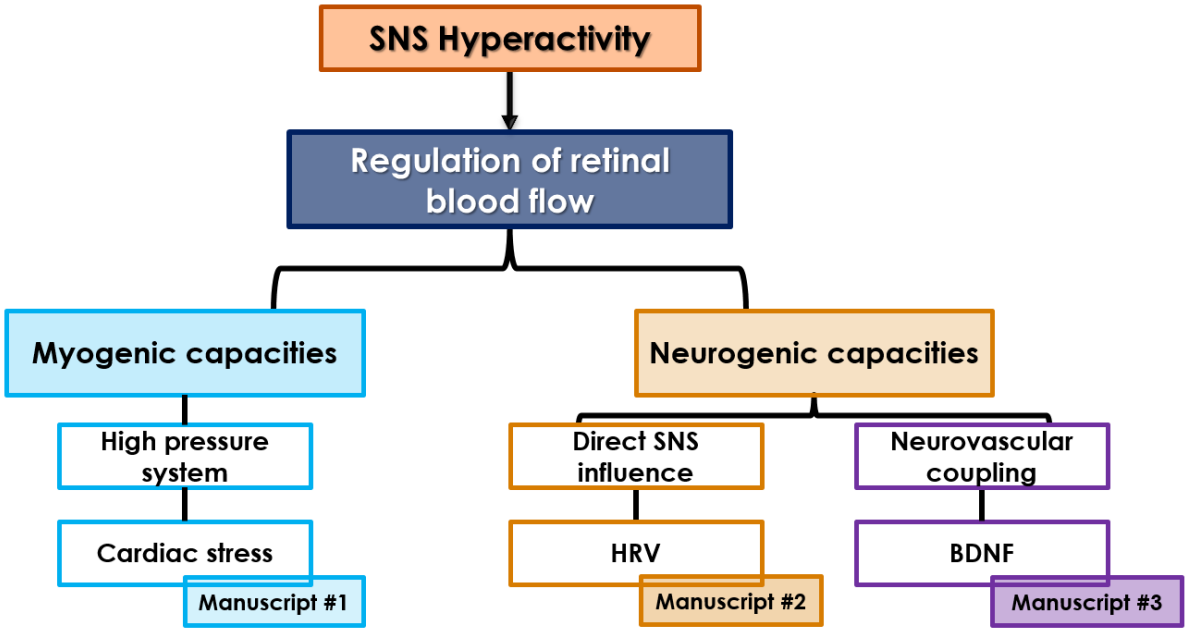

Figure 1.16: Proposed manuscript layout to assess the contributing influence of SNS hyperactivity on retinal vessel dynamics

Research questions that arise from the current literature:

- How does systemic cardiac stress, cTnT and NT-proBNP, influence retinal microvascular structure and function in Africans and Caucasians?
- How will cardiac stress markers, cTnT and NT-proBNP, be useful in predicting stroke risk within a sub-clinical, epidemiological setting in Africans and/or Caucasians?
- Does the SNS impact on the dynamic changes in retinal microvasculature?
- Is retinal autoregulation influenced by sustained SNS hyperactivity?
- Can FLIP be defined as a stressor that justifiably elicits a SNS-driven response?
- Will the most abundant neurotrophic factor in the retina, BDNF, be impacted by sustained SNS hyperactivity and relate to retinal vascular dynamics in Africans and/or Caucasians?
- Will BDNF levels translate to stroke risk within an epidemiological setting?
- THEREFORE will SNS-related markers (cTnT, NT-proBNP, BDNF) translate to stroke risk?

7. Aims

The primary aim of this study was to evaluate the possible influence of SNS hyperactivity in the brain-heart axis with a focus on the retinal vasculature's structure and function within a South African, bi-ethnic cohort. The translational value of SNS hyperactivity in these respective relationships (cardiac stress x retinal vasculature structure and function; HRV x retinal vasculature function; BDNF x retinal vascular function) and individual variables' (wider retinal venules, AV-nicking, cTnT, NT-proBNP and BDNF levels) relation to specifically stroke risk, was to be determined. The SABPA study was ideal to address these aims, as it is the only study in Sub-Saharan Africa which is designed to assess SNS activity and investigate the brain-retina-heart link, in a bi-ethnic cohort.

7.1. Comprehensive aims of each manuscript

7.1.1. Retinal vasculature reactivity during flicker-light-provocation, cardiac stress and stroke risk in Africans: The SABPA study

The aims of the first manuscript were to examine whether Africans, rather than Caucasians will display associations between disturbed retinal vessels a) structure (narrower arterioles and wider venules) and b) attenuated function (attenuated dilation and constriction) with SNS-associated cardiac stress markers (higher cTnT and lower NT-proBNP systemic levels). These associations may be translated to a higher stroke probability risk in Africans – thereby contributing to establishing a brain-retina-heart.

7.1.2. Heart rate variability, the dynamic nature of the retinal microvasculature and cardiac stress: Providing insight into the brain-retina-heart link: The SABPA study

The second manuscript built on the results of the first published manuscript (where a retina-heart link was established). The second manuscript would be the first to examine the retinal vessel structure and function, HRV time-and-frequency domain parameters during FLIP and cTnT to provide further evidence in support of the brain-retina-heart link. Due to the increased SNS tone well-documented in the SABPA African group, we aimed to determine whether FLIP-induced changes in HRV, compatible with increased SNS activity and tone and/or vagal withdrawal, will relate to altered retinal dynamics (attenuated dilation and constriction) and higher systemic levels of cTnT, indicating cardiac stress, specifically in Africans. Therefore, establishing a more clearly defined brain-retina-heart link.

7.1.3. Low Brain-derived-neurotrophic-factor reflects attenuated retinal vascular functionality and increased stroke risk: The SABPA study

Finally, the third manuscript's aims were built on the results of both the previous manuscripts. This study was to be the first to examine systemic levels of BDNF and relate it to retinal vasculature function during FLIP. Due to the well-documented stroke risk and attenuated arteriolar functionality in response to FLIP, in the SABPA African group, we aimed to determine whether lower systemic levels of BDNF will be associated with attenuated retinal arteriolar dilation and constriction during FLIP. Further, we aimed to determine whether sustained SNS hyperactivity may influence BDNF levels over 3 years and relate this influence to retinal arteriolar dynamics – indicative of disturbed neurovascular coupling. Additionally, we aimed to establish stroke risk related to low BDNF levels, according to ethnicity. Finally, to reinforce the brain-retina-heart link.

8. Hypotheses

Considering the available literature, and bearing in mind the aims of the current study, the following main hypotheses were proposed: Due to the SNS hyperactive status reported in Africans, we hypothesise that the African group will present with narrower retinal arterioles, wider retinal venules and attenuated retinal vascular responses during FLIP. These retinal vascular structural and functional parameters will associate with increased cardiac stress, SNS hyperactivity and lower BDNF levels in Africans exclusively. The overall sustained SNS hyperactivity will facilitate an increased susceptibility to ischemic stroke and myocardial ischemia in the African group, compared to their Caucasian counterparts.

8.1. Comprehensive hypotheses of each manuscript

8.1.1. Retinal vasculature reactivity during flicker-light-provocation, cardiac stress and stroke risk in Africans: The SABPA study

As the Africans presented a greater hypertensive prevalence profile than Caucasians, we hypothesized:

- that smaller arteriolar calibres, wider venular calibres, arteriovenous-nicking (AV-nicking) and diminished arteriolar and venular dilation during FLIP, will be associated with increased cardiac stress (cTnT and NT-proBNP) in Africans; and
- that these cardiac stress-retinal associations (cTnT, NT-proBNP, wider venules and AV-nicking) will individually reflect an increased stroke risk in Africans.

8.1.2. Heart rate variability, the dynamic nature of the retinal microvasculature and cardiac stress: Providing insight into the brain-retina-heart link: The SABPA study

Due to the increased SNS tone well-documented in the SABPA African group, we hypothesised:

- that FLIP-induced changes in HRV, compatible with increased SNS activity and tone and/or vagal withdrawal will relate to altered retinal dynamics (attenuated dilation and constriction) in Africans; and
- that FLIP-induced changes in HRV will inversely associate with higher levels of cTnT, indicating SNS influence on sustained cardiac stress, specifically in Africans.

8.1.3. Low Brain-derived-neurotrophic-factor reflects attenuated retinal vascular functionality and increased stroke risk: The SABPA study

Due to the well-documented SNS hyperactivity and stroke risk, in the SABPA African group, we hypothesise:

- that low systemic levels of BDNF will be associated with attenuated retinal arteriolar dilation and constriction during FLIP, specifically in Africans; and
- that an increased stroke risk will relate to lower BDNF levels, especially in the African group.

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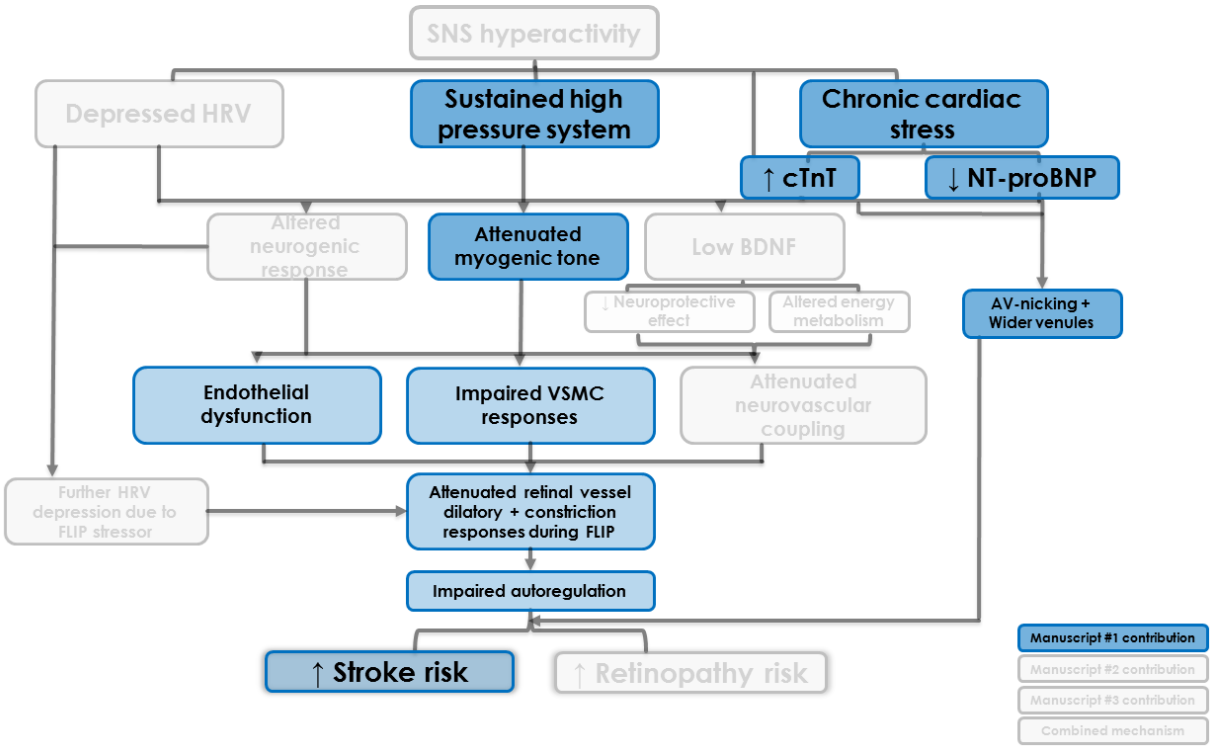
Chapter 2: Manuscript 1

Retinal vasculature reactivity during flicker-light-provocation, cardiac stress and stroke risk in Africans: The SABPA study

In this manuscript, the associations between retinal vessel structure-functionality and cardiac stress markers [cardiac troponin T (cTnT), amino-terminal B-type natriuretic peptide (NT-proBNP)] were assessed. We then further translated these retina-heart relationships to stroke risk as a clinical inference. cTnT levels were similar in Africans and Caucasians, whereas NT-proBNP levels were lower in Africans. In Africans, a reduced arteriolar calibre and attenuated arteriolar dilation during FLIP was associated with higher cTnT. Their larger retinal-venular calibre and attenuated arteriolar dilation during FLIP were associated with lower NT-proBNP. Again, exclusively in Africans, increased cardiac stress, wider venular calibres and retinal arteriovenous-nicking, predicted an increased 10-year stroke risk. None of these associations were evident in the Caucasian group. Therefore, observable changes in the retinal vasculature may serve as markers for the identification and prediction of cardio-systemic and cerebral vascular morbidities and risks – thereby establishing a brain-heart link.



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Topics	Neurosciences, Neurology, Cardiology, Neurosurgery, Vascular surgery
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Retinal vasculature reactivity during flicker-light-provocation, cardiac stress and stroke risk in Africans: The SABPA study

Short title: Dynamic retinal vessel responses, cardiac troponin T, NT-proBNP and stroke risk

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Abstract

Structural and functional similarities exist between the retinal, cerebral and, as previously suggested, the coronary vasculature. Retinal microvascular structure and functionality (in response to flicker-light-induced-provocation (FLIP)) may relate to coronary artery disease risk and possible stroke risk. We investigated associations between retinal vessel structure-functionality and cardiac stress markers [cardiac troponin T (cTnT), amino-terminal B-type natriuretic peptide (NT-proBNP)] to translate these retina-heart relationships to stroke risk. We included 317 African and Caucasian teachers' (aged 23-68 years), who participated in the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study. Fasting plasma and serum samples for cTnT and NT-proBNP were collected. Retinal vascular calibres were quantified from fundus images and dynamic retinal vessel calibre responses during FLIP. The University of California stroke risk score was applied to assess subclinical 10-year stroke risk. cTnT levels were similar in Africans and Caucasians, whereas NT-proBNP levels were lower in Africans. In Africans, a reduced arteriolar calibre and attenuated arteriolar dilation during FLIP was associated with higher cTnT ($p<0.01$). Their larger retinal-venular calibre ($p<0.02$) and attenuated arteriolar dilation during FLIP ($p<0.05$) were associated with lower NT-proBNP. Again, exclusively in Africans, increased cardiac stress, wider venular calibres and retinal arteriovenous-nicking, predicted an increased 10-year stroke risk with odds ratios of 1.57 (95% CI, 1.34; 1.68, $p=0.031$); 1.51 (95% CI, 1.26; 1.59, $p=0.002$); 1.10 (95% CI, 0.94; 2.85, $p=0.002$) and 1.06 (95% CI 0.83; 1.56, $p=0.052$), respectively. None of these associations were evident in the Caucasian group. Investigating the retinal vasculature may serve as a tool to approximate subclinical coronary and cerebral microvasculature damage or dysfunction. These cardiac-stress-retinal associations additionally predicted a greater stroke risk in the SABPA African cohort. Observable changes in the retinal vasculature may serve as markers for the identification and prediction of cardio-systemic and cerebral vascular morbidities and risks – thereby establishing a heart-brain link.

Key words: Retina; Dynamic retinal vessel responses; FLIP; NT-proBNP; cTnT; Stroke; Ethnicity

Abbreviations: AUC, area under the curve; DVA, dynamic vessel analyses; cTnT, cardiac troponin T; MC, maximum constriction; MD, maximum dilation; NT-proBNP, Amino-terminal pro-B-type natriuretic peptide; OR, odds ratio.

Introduction

Structural and functional similarities exist between the retinal, cerebral and, as previously suggested, the coronary vasculature [1]. Specifically, the retinal and cerebral vasculature show striking anatomical, functional and autoregulatory similarities – maintaining constant blood pressure (BP), despite changes in systemic BP [1]. The latter ensures adequate perfusion and the preservation of blood-ocular or blood-brain barriers [2]. However, the ability to maintain these autoregulatory capacities diminishes with increases in BP, such as during hypertension [3, 4]. The coronary arteries, possessing an elastic lamina, are tailored to accommodate frequent pulsatile changes, whereas the retinal and cerebral microvasculature's structure ensures adequate perfusion. Despite these anatomical differences, changes in retinal vessel calibres and functional responses to flicker-light-induced-provocation (FLIP) were present in patients suffering from coronary artery disease (CAD), compared to those without CAD [5].

A wider retinal venular calibre and reduced dilation in response to FLIP have been associated with an increased risk for concurrent and future cerebrovascular events [5-8], and CAD [5, 9]. Yet, the relationship between the structure and functionality (in response to FLIP) of retinal vessels with cardiac stress markers in the risk stratification for stroke, remains to be investigated. Furthermore, literature describing the overall retina-brain-heart link, remains limited [10].

Until recently, cardiac troponin T (cTnT) has been considered solely as a marker of cardiac apoptosis and necrosis [11, 12]. However, recent observations of detectable circulating cTnT levels in the general population have initiated a paradigm shift. Alternative mechanisms for cTnT release have been investigated, including increased myocardial stress (cardiac stress), due to increased pressure and/or volume load [11, 12]. In addition, small increases in cTnT levels have been linked to endothelial dysfunction and small vessel disease (cerebral and coronary), rather than myocardial damage exclusively [13, 14]. This prompts the question whether cTnT can be linked to the cerebral microvasculature and subsequent stroke risk [15].

A recent investigation established a positive relationship between cTnT and amino-terminal pro-B type natriuretic peptide (NT-proBNP) [16]. NT-proBNP is a recognised dynamic marker of cardiac stress [17], which is also functionally linked to retinal epithelial cells, glial cell regulation [17, 18] and, recently, with retinal microvascular damage [15]. Lower levels of NT-proBNP are associated with early microvasculature changes, including loss of endothelial integrity [20], hemodynamic modifications and reduced densities of both coronary and cerebral microvasculature, signifying an increased risk for cerebrovascular events [21].

We thus aimed to examine associations between retinal vessel structure and functionality with cardiac stress markers, NT-proBNP and cTnT, in a bi-ethnic cohort from South Africa. Furthermore,

as the urban-dwelling Africans presented a greater hypertensive prevalence profile than Caucasians [22], we hypothesized that smaller arteriolar calibres, wider venular calibres, arteriovenous-nicking (AV-nicking) and diminished arteriolar and venular dilation during FLIP will be associated with increased cardiac stress, and an accompanying higher stroke risk in Africans.

Materials and Methods

Study design and participants

The participants were initially recruited as part of phase 1 of the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study. All participants included in phase 1 (2008-2009) were invited to take part in the follow-up or phase two. Of the initial 409 participants, 359 reported for the second phase of the study. We only included participants who took part in the second phase of the study, as no retinal measurements were taken during the first phase. The study sample comprised of urban male and female African and Caucasian teachers (N=359) from the North West Province, South Africa, aged between 23 and 68 years. The motivation behind the exclusive selection of teachers was to obtain a socio-economic equated sample from a similar working environment; cultural differences could however not be excluded. Additional exclusion criteria for this study included epilepsy (n=1) and unsuccessful retinal vessel recordings (n=41). Regarding retinal vessel reactivity measurements in response to FLIP, only individuals with a quality score of equal to or higher than 2.5 were included (Supplementary methods). The majority of the unsuccessful retinal measurements were found in the African group. Finally, a total of 139 Africans and 178 Caucasians were included in the current study (sub-population N=317).

Ethical considerations

The SABPA study obtained ethical approval from the Health Research Ethics Committee (HREC) of the NWU and extended approval was granted for the second phase. Written informed consent was obtained from all volunteers prior to participation. All procedures and objectives were explained to the participants prior to their recruitment, and adhered to the applicable institutional guidelines and terms, as stated by the Declaration of Helsinki (2004).

General procedure of investigation

Clinical assessments were done over a two-day period during the second phase or follow-up – similar to baseline. Before 08h00 of the first clinical assessment day, participants were each fitted with an ambulatory blood pressure (ABPM) and 2-lead ECG monitor device (Cardiotens CE120®; Meditech CE120; Meditech, Budapest, Hungary). This device also obtained event BP prior and after retinal vessel assessments. A 24-hour standardized diet commenced and participants subsequently continued with their normal daily activities, reporting any peculiarities such as nausea, headaches, visual disturbances, palpitations, fainting, stress and physical activity, on the issued 24-hour diary cards. At 15h00, participants were transported to the NWU Metabolic Unit Research Facility for clinical measurements including the retinal vessel imaging.

Retinal vessel analyses

Participants abstained from caffeinated and alcoholic beverages, smoking, strenuous physical activity and food consumption for at least 1 hour prior to the measurements. They were familiarized with the experimental setup and a registered nurse examined them for acute angle anterior chamber glaucoma risk. Diastolic ocular perfusion pressure was calculated (Supplementary Information). The retinal vessel analyser (Imedos Systems UG, Jena, Germany) was used for digital fundus imaging with a Carl Zeiss FF450^{Plus} camera (Carl Zeiss, Meditech Jena, Germany) to perform dynamic and static retinal vessel analyses. Fifteen minutes prior to the measurement, a drop of Tropicamide (1% Alcon 1% tropicamide and 0.01% benzalkonium chloride (m/v)), was used to induce mydriasis in the right eye. If the right eye was not suitable, the left eye was used. Dynamic vessel analysis with flicker stimulation was performed first, followed by image capturing for static vessel analysis. In static vessel analysis we calculated the central retinal artery and vein equivalent (CRAE and CRVE respectively) and subsequently determined the arteriovenous ratio (AVR) as previously described in the SABPA study [23]. The presence of retinopathy and AV-nicking was determined by a registered ophthalmologist from a colour retinal image. (See Supplementary Methods, ***Appendix A***).

For dynamic vessel analysis a standard flicker protocol by IMEDOS Systems was used. During FLIP, the duration of the baseline was 50 seconds, followed by a 20-second flicker period and an 80 second recovery (also referred to as second baseline) period. There were 3 flicker cycles in total, lasting an added total of 350 seconds for the entire measurement. The camera was set at a 30°degree angle with the participant focusing on the tip of a fixation rod, and an artery and vein segment (as long as possible) were primarily selected in the upper or lower temporal quadrant of the fundus image. The quality of the FLIP measurements for each participant were assessed subjectively using a newly developed, previously described, scoring method and extensively described in the Supplementary Methods (*Smith W, Vilsert W, Kotliar K, 2019*).

Absolute vessel diameters (measured in standardized measuring units (MU)) of the vessel segment was selected for each measurement. Each were calculated individually as the median value over the last 30 seconds of the first baseline phase prior to FLIP. Parameters derived from the smoothed averaged curve during FLIP, used in the current study, included: 1) the percentage maximal dilation in response to FLIP ($MD_{arteriole}$); 2) The area under the reaction curve during FLIP (AUC) was determined for arteries and veins and calculated as the percentage change per second (%.s). The latter provided information on the curve form during FLIP (0-20s), and theoretically describes the longevity, time and intensity of the vessel's response. For the values under the 100%-line the area was negative; 3) The percentage absolute maximal constriction after FLIP (MC) was the minimum value occurring after maximum FLIP-induced dilation and expressed a percentage from baseline.

Biochemical analyses

Fasting blood samples were obtained from the antebrachial vein branches of each participant's dominant arm with a sterile winged infusion set, by a registered nurse. Blood samples were dealt with in accordance with the standardised protocol, and serum and plasma samples were frozen at -80˚C until analysed in duplicate. Cotinine values were determined by means of a homogeneous immunoassay with an automized Modular Roche method (Switzerland). γGT and ultra-high sensitivity C-reactive protein (hs-CRP) were analysed with the Konelab™20i (ThermoScientific, Vantaa, Finland). Tumour necrosis factor-alpha (TNF-α) was measured via a quantikine HS Elisa Human serum TNF-alpha Immuno-assay (R&D Systems, Minneapolis, MN USA); Inter (15%) and intra (17.8%) assay variability. Glycated haemoglobin (HbA_{1c}) EDTA Whole blood HbA_{1c} and serum cholesterol and high density lipoprotein (HDL) were determined with a turbidometric inhibition immunoassay and a homogeneous enzymatic colorimetric assay respectively (Integra 400 plus, Roche, Switzerland). NT-proBNP and cTnT were measured via an electrochemiluminecence method on the Roche® e411 (Roche®, Basel, Switzerland). Inter-batch variability 4.6%; intra-batch variability 4.2%. Below detectable limit cTnT values (31.3% of all cTnT analyses, N=104), were logarithmically calculated according to the method developed by [24].

Cardiovascular risk indicators

The 10-year UCLA risk composite score included gender, SBP, hypertensive drugs, diabetes, smoking habit, perfusion deficits, atrial fibrillation and electrocardiography (ECG) left ventricular hypertrophy (American Heart and Stroke certified UCLA Medical Centre, Primary Stroke Centre, Santa Monica, Los Angeles, USA). Medium to high stroke probability was termed as scores of 5.2 and greater. Additionally we applied recently defined cTnT cut-points predictive of clinical 24H hypertension. These cut-points were defined as cTnT ≥4.2ng/mL for the total African group and NT-proBNP levels below the age- and gender-specific reference values [25-27].

Statistical analyses

Statistica version 13.3 (TIBCO Software Inc., Palo Alto, USA, 2018) was used for data analyses. Kolmogorov-Smirnov tests assessed normality of all variables. Logarithmically transformed γGT, CRP, cotinine and HbA_{1c} levels were used in correlation models. Characteristics between ethnic groups were calculated with *t*-tests. Chi-square (X^2) statistics were used to determine proportions and prevalence data. *A priori* covariates included age, body surface area (BSA), cotinine, γGT, HbA_{1c}, total cholesterol/HDL cholesterol ratio, TNF-α, hypertensive/diabetic retinopathy and 24-hour pulse pressure [23]. Single two-way ANCOVAs determined ethnic x gender differences for all cardiac stress and retinal vessel markers, independent of *a priori* selected co-variates. One-way ANCOVA was used to determine the least square mean difference in response markers between ethnic groups,

independent of *a priori* selected covariates. Multivariate linear regression analyses were used to determine associations between Retinal Vessel Analyses (RVA) parameters and cardiac stress markers in several models. The dependent variables included 1) structure: CRAE, CRVE, AVR and 2) reactivity/functionality during FLIP: arteriole maximum constriction (MC), maximum dilation (MD), area under the curve $(AUC)_{arteriole}$ and venular (AUC_{venule}), both during FLIP. Independent variables included NT-proBNP, cTnT, along with the *a priori* selected covariates in all models. Additional adjustments were made for CRVE in the CRAE models and vice versa. For all the aforementioned analyses, significance was set at $p<0.05$ (two-tailed) and the F to enter was fixed at 2.5 in regression models. Odds ratios (OR) (95% confidence intervals (CI)), were computed in several models to determine the probability of a brain-heart link to predict stroke risk. Hence the following was calculated 1) the probability of cardiac stress [(cTnT ≥ 4.2ng/mL; NT-proBNP below the age and gender specific reference value [25-27]; and 2) wider retinal venular calibres (CRVE ≥ 248 MU [22-23]) and AV-nicking (Henderson et al, 2011), to predict medium-high UCLA 10-year stroke risk.

Sensitivity analyses

Forward stepwise regression analyses with the same set of covariates were repeated in several models in both ethnic groups. Analyses were computed by excluding participants with diabetes (n= 36) and those using angiotensin converting enzyme inhibitors (n= 52). None of these variables statistically significantly ($p<0.05$) influenced the outcome.

Results

Interaction testing revealed that significant differences existed between Africans and Caucasians, but not between men and women, independent of *a priori* selected covariates for NT-proBNP ($F_{1, 317}$ = 4.0, p=0.046); cTnT ($F_{1, 317}$ = 27.1, p<0.001); Arteriole MD (%) ($F_{1, 308}$ = 19.27, p<0.001); and Venular MD (%) ($F_{1, 308}$ = 20.65, p=0.024).

Baseline characteristics

Table 2.1 displays a comparison between the characteristics in ethnic groups. The Caucasian group were slightly older. Africans displayed lower BSA, but a poorer cardio-metabolic profile with higher CRP, HbA_{1c} and cholesterol levels, compared to their Caucasian counterparts (p<0.05). Caucasians revealed higher NT-proBNP (p<0.001) levels, but similar cTnT values were observed between ethnic groups. The African group had higher 24-hour BP and PP, pre- and post-FLIP BP (p<0.001), IOP (p<0.001) and DOPP (p=0.010) values, hypertensive/diabetic retinopathy prevalence of 86% compared to 39% in Caucasians and prevalence of AV-nicking of 64% vs 21% in Caucasians.

Unadjusted and adjusted retinal calibres

In **Table 2.2**, the African group revealed a higher CRVE (p<0.001) and smaller ARV (p<0.001). A comparison between dynamic retinal vessel parameters revealed that both arteriolar and venular maximal dilation in response to FLIP were greater in Africans (p<0.05). The $AUC_{arteriole}$ confirmed the significantly higher arteriolar vessel response during FLIP (p<0.05) in Africans. NT-proBNP and cTnT trends remained similar to the unadjusted analyses in both ethnicities.

Static retinal calibres and cardiac stress markers

Table 2.3 reveals that in Africans CRAE was inversely associated with cTnT, whereas CRVE was positively and ARV inversely associated with NT-proBNP (*p*=0.019). None of these associations were significant in the Caucasian group.

Retinal vessel calibres in response to FLIP and cardiac stress markers

Concerning vessel responses to FLIP (**Table 2.3**), only arteriolar maximum dilation was inversely associated with cTnT (*p*=0.026) in the African group. In addition, $AUC_{arteriole}$ during FLIP, depicting the longevity, length and duration of vessel reactivity, was inversely related to cTnT and NT-proBNP (*p*=0.049).

In **Figure 2.1A**, the 10-year stroke risk probability in Africans is illustrated in individuals with cTnT levels of ≥4.2ng/mL, NT-proBNP levels below the normal reference. In Africans a medium-high 10-year stroke risk was associated with an OR of 1.51 (95% CI, 1.04; 1.44, p=0.002) and 1.57 (95% CI,

1.26; 1.59, p=0.032), for increased cTnT levels and for NT-proBNP below reference values respectively. In **Figure 2.1B** retinal stroke markers, CRVE ≥ 248 MU [OR of 1.10 (95% CI, 0.94; 2.85), p<0.001] and AV-nicking [OR 1.06 (95% CI, 0.83; 1.56) p=0.052] predicted a medium-high 10-year stroke risk. Excluding participants with diabetes and those using angiotensin converting enzyme inhibitors, did not influence the outcome.

Table 2.1: Baseline characteristics between ethnicities

Variable	Africans (N=139)	Caucasians (N=178)	*p*-value
Lifestyle and Biochemical measurements			
Age, years	48 ± 8	50 ± 10	0.037
BSA (m^2)	1.94 ± 0.24	2.04 ± 0.29	0.001
Physical activity (kcal/day)	3318.47 ± 1257.10	3462.78 ± 1633.80	0.356
*Serum cotinine (ng/ml)	32.61 (18.60, 36.00)	21.63 (10.45, 31.00)	0.242
*γGT (U/L)	37.55 (22.30 ,67.30)	18.00 (11.90, 29.60)	<0.001
*CRP (mg/L)	4.61 (2.00, 9.60)	2.59 (0.99, 3.98)	<0.001
TNF-α	2.48 ± 2.18	2.74 ± 1.68	0.198
$HbA1_c$ (%)	6.19 ± 1.33	5.59 ± 0.67	<0.001
Total cholesterol: HDL	4.91 ± 1.67	4.43 ± 1.48	0.005
cTnT (pg/mL)	4.68 ± 3.71	5.04 ± 1.15	0.349
NT-proBNP (pg/mL)	61.85 ± 68.44	80.98 ± 72.17	<0.001
Cardiovascular measurements			
24H ABPM SBP (mmHg)	139 ± 18	128 ± 11	<0.001
24H ABPM DBP (mmHg)	88 ± 11	79 ± 8	<0.001
24H ABPM PP (mmHg)	52 ± 10	48 ± 7	<0.001
DVA pre-FLIP SBP (mmHg)	141 ± 21	133 ± 15	<0.001
DVA pre-FLIP DBP (mmHg)	88 ± 13	83 ± 11	<0.001
DVA post-FLIP SBP (mmHg)	139 ± 19	132 ± 13	<0.001
DVA post-FLIP DBP (mmHg)	88 ± 13	83 ± 10	<0.001
IOP (R) (mmHg)	16.51 ± 4.14	14.95 ± 3.42	<0.001
DOPP (R) (mmHg)	71.68 ± 13.31	68.12 ± 10.77	0.007
Static retinal parameters			
CRAE (MU)	149.68 ± 1.20	151.01 ± 1.00	0.317
CRVE (MU)	249.19 ± 1.80	236.68 ± 1.60	<0.001
ARV	0.60 ± 0.06	0.64 ± 0.04	<0.001

Dynamic retinal parameters			
Arteriole MD (%)	4.11 ± 2.46	3.51 ± 1.94	0.015
Arteriole MC (%)	-1.54 ± 1.17	-1.70 ± 1.33	0.272
Venule MD (%)	4.72 ± 2.35	4.10 ± 1.85	0.008
AUC during FLIP			
AUC $FLIP_{arteriole}$(%.s)	57.88 ± 39.15	50.28 ± 33.08	0.061
AUC $FLIP_{venule}$(%.s)	58.51 ± 35.66	49.54 ± 26.59	0.010
History			
MI events, N (%)	1 (0.58)	1 (0.54)	0.959
Hypertensive, N (%)	112 (80.58)	63 (35.39)	<0.001
Diabetic, N (%)	27 (19.42)	10 (5.62)	0.003
Atrial fibrillation, N (%)	3 (2.16)	3 (1.69)	0.959
NT-proBNP below reference value, N (%)	85 (61.15)	104 (58.42)	0.052
Hypertensive/ Diabetic Retinopathy, N (%)	120 (86.33)	69 (38.76)	<0.001
Arteriovenous nicking, N (%)	89 (64.03)	38 (21.35)	<0.001

*Data presented as median and interquartile ranges. Data expressed as arrhythmic mean ± SD. Where: N, number of participants; %, percentage change; CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha; HbA_{1c}, glycated haemoglobin; γGT, gamma glutamyl transferase; NT-proBNP, N-terminal pro-brain natriuretic peptide; cTnT, cardiac Troponin T; ABPM, ambulatory blood pressure measurement; SBP, systolic blood pressure; DBP, diastolic blood pressure; IOP, intra-ocular perfusion pressure; DOPP, diastolic ocular perfusion pressure; FLIP, Flicker-light-induced-provocation; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; MD, maximum dilation during FLIP; MC, maximum constriction during FLIP; AUC during FLIP; MI, myocardial infarction.

Table 2.2: Comparison of static and dynamic retinal vessel calibres and cardiac stress markers between ethnicities, independent of *a priori* covariates

Variable	Africans (N=139)	Caucasians (N=178)	*p* value
Static vessel analyses			
CRAE (MU)	149.78 ± 1.09	150.94 ± 0.95	0.287
CRVE (MU)	248.39 ± 1.71	237.49 ± 1.50	<0.001
AVR	0.61 ± 0.004	0.64 ± 0.004	<0.001
Dynamic vessel analyses			
Arteriolar MD (%)	3.98 ± 0.19	3.58 ± 0.16	0.030
Arteriolar MC (%)	-1.47 ± 0.11	-1.72 ± 0.09	0.190
Venular MD (%)	4.73 ± 0.17	4.05 ± 0.16	0.014
AUC data			
FLIP $AUC_{arteriole}$	55.49 ± 3.10	51.47 ± 2.70	0.111
FLIP AUC_{venule}	58.02 ± 2.53	49.42 ± 2.28	0.027
Biochemical markers			
cTnT (pg/mL)	4.80 ± 0.27	4.98 ± 0.25	0.344
NT-proBNP (pg/mL)	64.86 ± 5.64	79.56 ± 5.17	0.009

Data expressed as an arithmetic mean (± SE). *A priori* covariates included age, body surface area, cotinine, gamma glutamyl transferase, $HbA1_c$, total cholesterol:HDL ratio, hypertensive/diabetic retinopathy and 24H pulse pressure. For NT-proBNP models, thyroxin and triiodothyronine were added. Where FLIP, flicker-light-induced-provocation; AVR, artery-to-vein ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; MD, maximum dilation; MC, maximum constriction; AUC, area under the curve ; cTnT, cardiac troponin T; NT-proBNP, N-terminal pro-brain natriuretic peptide.

Table 2.3: Independent associations between static and dynamic retinal vessel parameters and cardiac stress markers in Africans.

Africans (N= 139)			
	CRAE (MU)	**CRVE (MU)**	**AVR**
	ß (95% CI)	**ß (95% CI)**	**ß (95% CI)**
Adjusted R^2	0.37	0.24	0.38
cTnT (pg/mL)	-0.21 (-0.38, -0.04)	NS	NS
	p=0.012		
NT-proBNP (pg/mL)	NS	0.20 (0.039, 0.36)	-0.25 (-0.41, -0.09)
		p =0.019	*p*=0.003
Dynamic retinal parameters in response to FLIP:			
	Arteriole MD (%)	**$AUC_{arteriole}$**	**Venular MD (%)**
	ß (95% CI)	**ß (95% CI)**	**ß (95% CI)**
Adjusted R^2	0.15	0.34	0.03
Age (yrs)	-0.30 (-0.37, -0.21) *p*=0.001	-0.33 (-0.41, -0.25) *p*<0.001	-
cTnT (pg/mL)	-0.19 (-0.35, 0.03)	-0.12 (-0.29, 0.05)	-
	p=0.026	*p*=0.081	
NT-proBNP (pg/mL)	-	-0.12 (-0.30, -0.06)	NS
		p=0.049	

No significant associations were observed for vein dilation or AUC_{vein} , during FLIP, with any of the cardiac stress markers. All analyses were adjusted for age, cotinine, TNF-α, total cholesterol: HDL-cholesterol ratio, glycated haemoglobin (HbA_{1c}), 24H PP, hypertensive/ diabetic retinopathy. Additional adjustments were made for CRVE in the CRAE model and vice versa. For NT-proBNP models, thyroxin and triiodothyronine were added as covariates. Abbreviations: ns, not significant; CI, confidence interval; AVR, artery-to-vein ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; FLIP; flicker-light-induced-provocation; MD, maximum dilation during FLIP; MC, maximum constriction during FLIP; AUC during FLIP; cTnT, cardiac troponin T; NT-proBNP, N-terminal pro-brain natriuretic peptide. No statistical significant associations existed between MC of the artery and the cardiac stress markers.

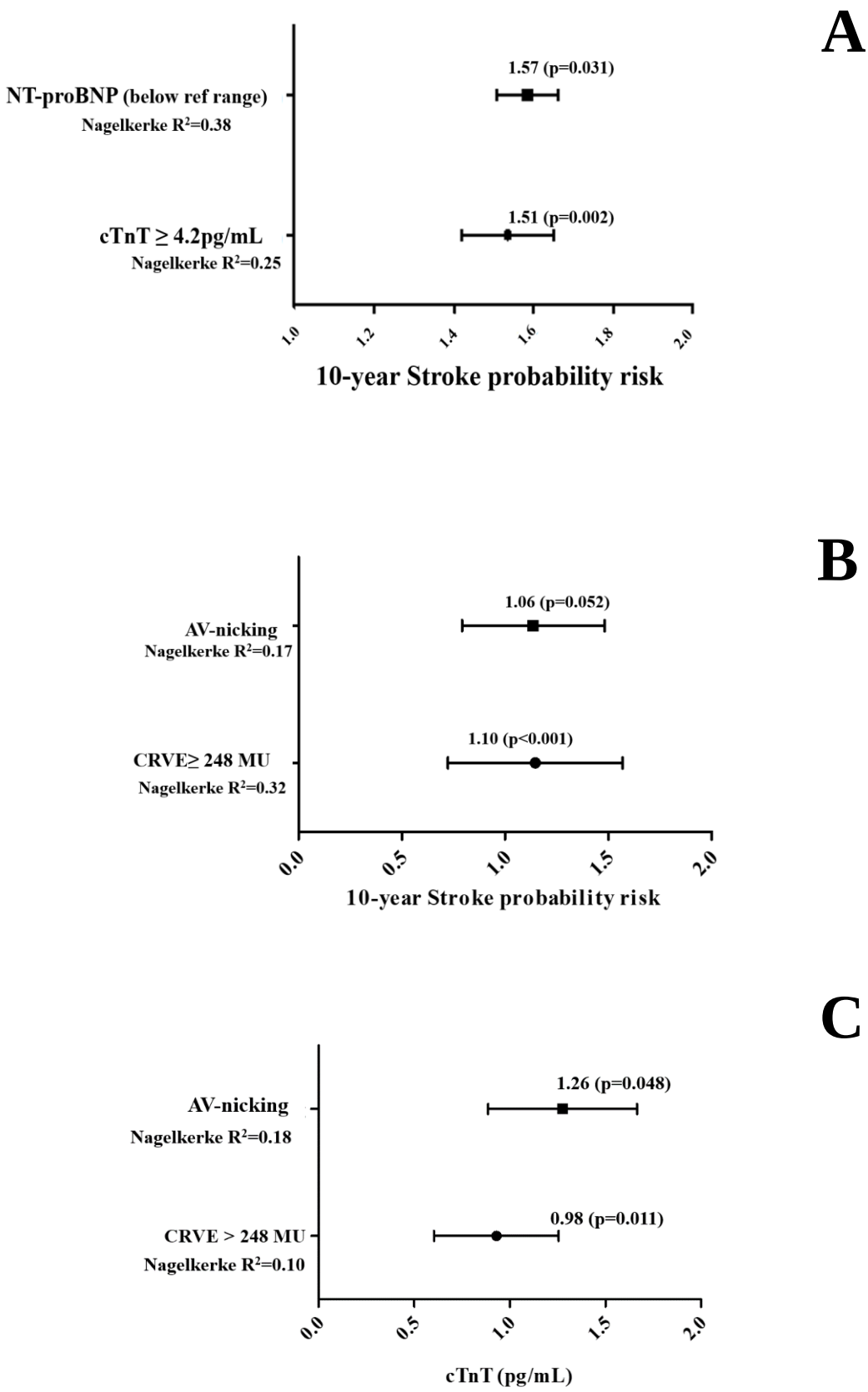

Figure 2.1: Odds ratios (95%CI) predicting A) Probability of medium-high 10-year stroke risk with a cTnT level of ≥4.2pg/mL and NT-proBNP levels below the age-and-gender specific reference values for Africans; B) Probability of medium-high 10-year stroke risk with AV-nicking and CRVE

> 248 MU in Africans.; C) Probability of increased systemic cTnT levels with AV-nicking and CRVE > 248 MU in Africans (*Supplementary Figure*). Strength of modelled relationships depicted as Nagelkerke^2 values (R^2).

*These odds ratios were exclusively present in Africans (N=107).

Discussion

The present study examined the relationship between retinal vessel structure and functionality (in response to FLIP), cardiac stress markers and stroke risk in a bi-ethnic population from South Africa. Our findings in Africans revealed that reduced retinal arteriolar calibres were associated with higher cTnT levels, whereas an attenuated arteriolar dilatory response to FLIP (MD and AUC_{artery}), was inversely associated with cardiac stress (both cTnT and NT-proBNP). Venular calibre were positively associated with NT-proBNP. Cardiac stress markers, wider venules and AV-nicking predicted an increased, medium-high subclinical stroke risk in Africans. Observable changes in the retinal vasculature and cardiac stress markers support the notion that these markers may serve as investigative tools for the prediction of cardio- and cerebral vascular risks and thereby establishing a more defined heart-brain link.

Static retinal calibres, cardiac stress markers, stroke risk and ethnicity

The African group exhibited higher ambulatory, pre- and post-FLIP BP, diastolic ocular perfusion pressure (DOPP) levels and 64% of participants with AV-nicking. Elevated resting BP levels have been associated with structural microvascular changes, directly relating to cardiac stress, stroke and hypertension. Hypertension may manifest as retinopathy, which is in its own right, predictive of stroke and CAD risk [8, 23, 28]. Complementary to the former, AV-nicking is a recognized phenomenon related to arteriosclerotic thickening of the small vessel walls and subsequent stroke risk [29]. Sustained high systemic BP, such as observed in the African group, alters perfusion pressures and adequate blood flow to peripheral tissues [23]. The modified hemodynamic responses observed during such high-pressure conditions, directly contribute to faulty retinal autoregulation, as the autoregulatory capabilities tend to vanish with increased BP and ocular pressures [1]. This sustained high-pressure system leads to a decrease in and eventual diminishing of myogenic control mechanisms (Bayliss effect), ultimately responsible for autoregulation [3, 4]. Here, we were able to link the retinal microvasculature (a surrogate for the brain microcirculation) to markers of heart function and structure, via cTnT and NT-proBNP, in Africans, although not in Caucasians. Smaller estimated size of the central retinal artery (CRAE) associated with higher cTnT. A smaller central retinal artery diameter and wider vein diameter (CRVE) links to a greater CVD and stoke-risk profile and reflects long-term exposure to high BP [30, 31]. Higher cTnT levels have also been identified as an independent stroke-risk marker and predictor of cerebral micro-bleeds [32, 33]. This prediction of cerebral micro-bleeds may imply a role of cTnT in other sub-clinical micro-vessel diseases. Indeed, our findings support this notion, as the relative risk of developing stroke in the subsequent 10 years was approximately 50% more increased in Africans when cTnT levels were above 4.2ng/mL (OR= 1.51). Overall, higher systemic levels of cTnT predicted AV-nicking and CRVE > 248 MU, in Africans (OR 1.26 and 0.98), respectively (**Figure 2.1 C**).

Yet another association that existed solely in the African group, is that of NT-proBNP with a wider venular calibre. This might indicate that an increased volume-load, due to pressure build-up in the venous system, and subsequent cardiac stress, albeit sub-clinical, possibly contributing to wider venular diameters, the eventual diminishing of myogenic control mechanisms and autoregulation, in an attempt to expand the accommodation capacity [34-36]. The latter modified hemodynamic response may also contribute to stroke risk, as its end-result lies in disrupting the autoregulatory competence of both the cerebral and retinal microvasculature. Our previous work supports this finding, in that increased diastolic ocular perfusion pressure, or hyperperfusion, was associated with an increased vein diameter [22]; a recognized risk predictor for stroke [8]. Currently we showed that a mean hypertensive state, higher cTnT, lower NT-proBNP levels and structural changes in retinal vasculature were all independently related to stroke risk.

Lower levels of NT-proBNP were directly linked to increased stroke risk in a North-American bi-ethnic population [20, 21]. Our findings thus support this observation with an increased 10-year stroke probability in Africans (OR= 1.57) existing when NT-proBNP levels were below the age- and gender-specific reference ranges. Additionally, a greater CRVE (≥248 MU) and AV-nicking predicted a slightly higher UCLA stroke-risk score in Africans (OR's = 1.10 and 1.06).

Dynamic retinal vasculature responses to FLIP, cardiac stress markers and stroke

The current study revealed contrasting ethnic associations of cTnT and the retinal arteriole's ability to maximally dilate in response to FLIP, possibly due to sustained high BP and the accompanying endothelial impairment. cTnT is associated with coronary microvascular- and endothelial function [37]. This introduces the possibility that a compromised hemodynamic state (sustained hypertension, volume-load, increased DOPP and increased ischemia) and accompanying endothelial dysfunction, additionally contributes to alterations in retinal vessel autoregulation. This might possibly be by way of pressure-alterations and/or desensitised responses to endothelial-linked messengers, such as nitric oxide [22]. Schneider et al [13] showed that cTnT does not only associate with general endothelial function, but specifically with dysfunction related to cerebrovascular pathology. Higher levels of cTnT associated with increased risk for vascular dementia. cTnT may possibly also contribute to sub-clinical small vessel disease affecting the retina (retinopathy), heart (CAD) [9] and the brain (stroke and dementia) [13]. To further link cTnT's role to stroke risk, we refer to the inverse association with retinal arteriolar dilation in the current study. A recent study conducted by Conzen [38] reported diminished retinal arteriolar dilation in patients with subarachnoid haemorrhage. This might not only support the attenuated arteriolar dilation response to FLIP with increased cTnT levels in Africans, but indirectly links a diminished arterial dilatory response to an increased risk for stroke. Additional cardiac stress markers (NT-proBNP) may relate to this dynamic profile, as literature regarding cTnT and the microvasculature is sparse.

The lower levels of NT-proBNP in Africans, accompanied by higher BP, may indicate and relate to early endothelial dysfunction and subsequent desensitisation to the vaso-reactive, cardio-protective effect of BNP. This scenario indicates that a pre-existent increase in cardiac stress might influence the dilatory ability of the arteriole during FLIP. This in turn, may affect the general auto-regulatory capacity of the retinal microvasculature. We carefully suggest that the arterioles may subsequently take a longer time to autoregulate during FLIP and may possibly affect the recovery of typical vessel diameter after FLIP. Retinal autoregulation is controlled by glial cell activity [28]. Glial cells not only regulate the release of BNP, but certain actions of glial cells are also controlled by the binding of BNP [18]. Expression of BNP has also been detected in astroglial cells, which cohesively function with the microvasculature [19]. Neurons, glial cells and the cerebral endothelium exhibit a unique relationship as they function as a cohesive unit, similar to that of the retinal microvasculature [38]. A diminished vessel response capacity and possibly impaired autoregulatory ability may additionally support the increased stroke risk observed at lower NT-proBNP levels. However, whether alterations in NT-proBNP levels precede and/or occur during ischemic stroke or perfusion deficits remains unknown. We therefore carefully suggest that in an already compromised high-pressure system, lower bio-availability of NT-proBNP may contribute to the impaired auto-regulatory ability of the retinal and cerebral microvasculature. This may either be due to pressure-induced endothelial dysfunction, or by way of the previously described glial cell interaction. The latter not only notably links the retinal vasculature to the cerebral circulation, but may also provide additional support for NT-proBNP as an independent indicator of increased stroke-risk probability.

Limitations and recommendations

Limitations of the current study may include the specific population (only including African and Caucasians teachers from one demographic area) as well as the cross-sectional design, which prevents identification of physiological mechanistic cause-and-effect relationships. Furthermore, as the risk profile differs distinctly between our African and Caucasian teachers, we are unable to evaluate whether the differences are ethnic specific, or whether the results are due to the observed higher risk in the African cohort. Unfortunately, the sample size of pair-matched risk profiles were too small to perform meaningful statistical analyses. Several investigations have identified increased psychosocial stress experienced by this vulnerable African cohort, as one of the main contributors to this observed higher risk profile [22, 23, 25, 26]. However, the exploration of psycho-social stress influences are not within the scope of the current investigation and we suggest assessing the effect of psychosocial stress on the structure and function of the retinal vasculature, cardiac stress markers and stroke risk. We further recommend future retinal vessel investigations to include beat-to-beat BP monitoring, as cardio-metabolic demands increase during the FLIP, and blood pressure variations will occur in order to maintain homeostasis.

Conclusion

Our findings in Africans revealed that reduced retinal arteriolar calibres were associated with cTnT, whereas an attenuated dilatory response to FLIP was inversely associated with both cTnT and NT-proBNP. A wider venular calibre was positively associated with NT-proBNP. These cardiac stress-retinal associations additionally predicted, albeit sub-clinically, an increased stroke risk in the SABPA-Africans. These results support the notion that structural and functional similarities exist between retinal, cerebral and coronary circulation. Observable changes in both the retinal vasculature and cardiac stress markers may serve as investigative tools for the identification, discovery and prediction of cerebrovascular risk.

Compliance with ethical standards

The SABPA study obtained ethical approval from the Health Research Ethics Committee (HREC) of the NWU and extended approval was granted for the second phase (Ethics number: **NWU-00036-07-S6)**. Written informed consent was obtained from all volunteers prior to participation. All procedures and objectives were explained to the participants prior to their recruitment, and adhered to the applicable institutional guidelines and terms, as stated by the Declaration of Helsinki (2004). Please also refer to uploaded SABPA Protocol article.

Conflict of interest

All authors declare no conflict of interest. Miss A Wentzel declares no conflict of interest, Prof L Malan declares no conflict of interest, Prof W Smith declares no conflict of interest, Prof Dr R von Känel declares no conflict of interest and Prof NT Malan declares no conflict of interest.

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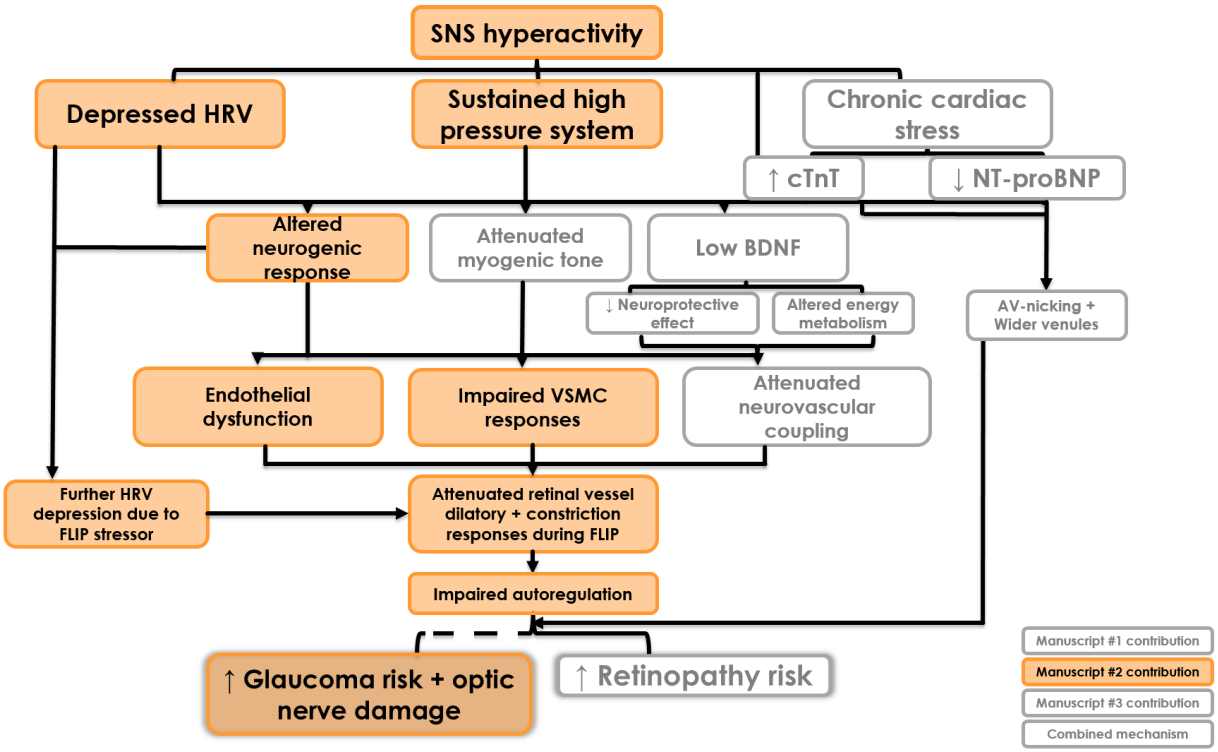
Chapter 3: Manuscript 2

Heart rate variability, the dynamic nature of the retinal microvasculature and cardiac stress: Providing insight into the brain-retina-heart link: The SABPA study

After the establishment of a retina-heart link in the first manuscript, we investigated the controversial role of autonomic nervous system modulation on retinal vascular dynamics and structure. We investigated associations between the retinal vasculature's structure and functionality, cardiac stress and classic markers of autonomic nervous system modulation, or heart-rate-variability (HRV) to provide evidence in support of the **brain**-retina-heart link. Africans had wider venules and attenuated time domain parameters during FLIP. Flicker light elicited increased sympathetic nervous system (SNS) modulation in this bi-ethnic cohort. In Africans, decreased HRV during FLIP accompanied greater arteriolar and venular responses and elevated systemic levels of cTnT, implying that the SNS exerted a significant effect on the smooth-muscle tone of the retinal vasculature, either directly or indirectly. This manuscript showed that disrupted retinal autoregulation may imply general autonomic nervous system dysfunction. Additionally, we showed that higher SNS reactivity may increase this population's risk for glaucoma and optic nerve damage. This exemplifies central control by the brain on all systemic regulatory functions, across all vascular beds.



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Heart rate variability, the dynamic nature of the retinal microvasculature and cardiac stress: Providing insight into the brain-retina-heart link: The SABPA study

Short title: Heart rate variability**,** dynamic retinal vessel responses, cardiac troponin T

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Abstract

Background and Aims: Decreased heart-rate-variability (HRV) indicates increased sympathetic nervous system (SNS) activity and modulation with a shift in the sympatho-vagal balance towards SNS predominance. Increased SNS activity may precede volume-loading hypertension, contribute to increases in cardiac troponin T (cTnT), endothelial dysfunction and small-vessel disease. Hence we investigated the retinal vasculature, HRV during flicker-light-induced-provocation (FLIP) and systemic cTnT, a marker of cardiac stress, to provide further evidence in support of the brain-retina-heart link.

Methods: Cross-sectional observations were obtained from a bi-ethnic cohort (N=264), aged 23-68 years. Fasting serum samples for cTnT were obtained. Retinal vascular calibres were quantified from mydriatic eye fundus images, and dynamic retinal vessel calibre responses were determined during FLIP. Frequency and time domain parameters of HRV were calculated during FLIP for each participant.

Results: Africans had wider venules and attenuated time domain parameters during FLIP. In Africans, inverse associations emerged between arteriolar dilation and both cTnT and root-square-mean-difference-of-successive-RR-intervals (rMSSD) (p=0.030), and between arteriolar constriction and both low-frequency expressed in normalised units (LFnu) (p=0.003) and high-frequency expressed in normalised units (p=0.021). In Africans, wider venules inversely associated with standard-deviation-of-successive-RR-intervals (SDNN) as well as with LFnu (p-0.009). An opposite profile was observed in Caucasians with both time- and frequency-domain parameters of HRV in relation to retinal vessel structure and function.

Conclusion: FLIP-elicited increased SNS activity and modulation in this bi-ethnic cohort. In Africans, decreased HRV during FLIP accompanied greater arteriolar and venular responses and elevated systemic levels of cTnT, implying that the SNS exerted a significant effect on the smooth-muscle tone of the retinal vasculature. Disrupted retinal autoregulation may imply general autonomic nervous system dysfunction; exemplifying central control by the brain on all systemic regulatory functions across different vascular beds.

Key words: Heart-Rate-Variability, Retinal vessel dynamics, Ethnicity, SNS hyperactivity, cardiac troponin T

Introduction

Controversy exists regarding the contribution of the autonomic nervous system (ANS) to the retinal microvasculature's autoregulatory capacities [1-3]. Lanigan and co-workers demonstrated that mean retinal vessel responses to systemic sympathetic stimulation were significantly reduced in sympathectomised eyes [3]. Decreased sympathetic nervous system (SNS) may then cause loss of tone and/ or altered hemodynamics of all microvascular beds, and such decreased SNS activity may occur as an integral feature of a general ANS dysfunction or dysregulation. Indeed, peripheral autonomic neuropathy was linked to the regulatory impairment of the retinal neurovascular complex, leading to altered retinal vessel responses [4]. However, whether ANS alterations influence retinal vessel responses during flicker-light-induced-provocation (FLIP), remains unclear. The ability of the ANS to make rapid adjustments to situational demands is evident in the control of heart rate.

Heart-Rate-Variability (HRV) is the most often employed, non-invasive assessment of ANS activity and modulation. HRV is the variation in the time interval between successive heart beats [5]. Reduced HRV indicates a shift in the sympatho-vagal balance towards SNS predominance [6]. This may be attributed to increased SNS activity/modulation, vagal withdrawal or both [6, 7]. A decrease in cardiac vagal tone accompanied impaired coronary microvascular function [6], a decrease in resting cerebral blood flow, and reduced metabolic activity [8]. Reduced HRV has also been observed in Africans during acute mental stress, although not in Caucasians [9, 10]. Reduced resting HRV was also observed in Afro-Americans – more so than in European Americans [8].

The SNS is directly activated in myocardial ischemia and small vessel diseases, but increased SNS activity also precedes these events [11]. Higher levels of the cardiac stress marker cardiac troponin T (cTnT) was linked to endothelial and microvasculature disturbances in hypertensive patients [12]. Increases in cTnT levels in Africans in response to acute mental stress was observed, compared to Caucasians [9]. Such acute stress-induced increases in cTnT might indicate a link between the SNS and cTnT release, as a link between cTnT and retinal vessel responses was recently established [13].

To our knowledge, no study has examined the time and frequency domain measures of HRV during FLIP in relation to dynamic changes in the retinal vasculature and cTnT. We investigated retinal vessel structure and function, HRV time- and frequency-domain parameters during FLIP and cTnT to provide further evidence in support of the brain-retina-heart link. Due to the increased SNS tone, well-documented in the SABPA African group [9, 14-17], we hypothesised that FLIP-induced changes in HRV, compatible with increased SNS activity and tone and/or vagal withdrawal, will relate to altered retinal dynamics and higher levels of cTnT, indicating cardiac stress – specifically in Africans.

Materials and Methods

Study design and participants

For the first wave, all teachers enrolled in 43 schools (N=2170) of the Dr Kenneth Kaunda Education District, North West Province, South Africa, were invited to participate (**Figure 3.1**). Power analyses were performed for the SABPA study cohort by using previous studies for autonomic dysfunction to obtain relevant effect sizes based on differences in biological profiles. Resulting sample sizes of 50 – 416 enabled explanation of biological differences with a statistical power of 0.8, and level of significance of 0.05. The target population included urban-dwelling well-educated Black (African) and White African (Caucasian) male and female teachers. This exclusive selection of teachers ensured a socio-economic-education equated sample from a similar working environment; cultural differences could not be excluded. All volunteering teachers had medical aid benefits and were screened to meet study eligibility criteria during the recruitment phase (Figure 3.1). Those complying, formed the respondent group of 409, but those not complying, formed the non-respondent group (N=62) [18]. Data are currently available for 409 teachers of phase 1 and all were invited to partake in phase 2 from which 359 were followed up in phase 2. The participants voluntarily took part in the study and were initially recruited as part of phase 1 of the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study. All participants included in phase 1 (2008-2009) were invited to take part in phase two (2011-2012). Of the initial 409 participants, 359 reported for the second phase of the study. Only participants, who took part in the second phase of the study, were included for this study, as no retinal measurements were taken during the first phase. The study sample comprised urban male and female African and Caucasian teachers (N=359) aged 23-68 years from the North-West Province, Kenneth Kaunda educational district, South Africa. This cohort and selection is fully described elsewhere [18]. All teachers from different schools were informed about the aims of the study and written informed consent was obtained before their inclusion in the study. As they were volunteers, any participant was free to withdraw at any stage of the investigation. Specific exclusion criteria for phase 1 of the SABPA study were however: alpha/beta blocker users, any psychotropic drug (e.g. anti-depressants), pregnant or lactating women, blood donation or vaccination 3 months prior to the investigation and tympanum temperatures of higher than 37.5ºC.

Additional exclusion criteria for the current study were epilepsy (n=1), arrhythmia (n=17), unsuccessful or poor retinal vessel recordings (n=41) and HRV data during FLIP (n=46). The majority of unusable retinal measurements were found in the African group, where poor images were obtained of participants who did not want to continue with the measures or could not remain in a sitting position without moving. Regarding retinal vessel reactivity measurements in response to FLIP, only individuals with a quality score of equal or higher than 2.5 were included (*Supplementary methods*). The majority of the unsuccessful retinal measurements were found in the African group.

Finally, a total of 127 Africans and 137 Caucasians were included in the current study (sub-population N=264) (**Figure 3.1**).

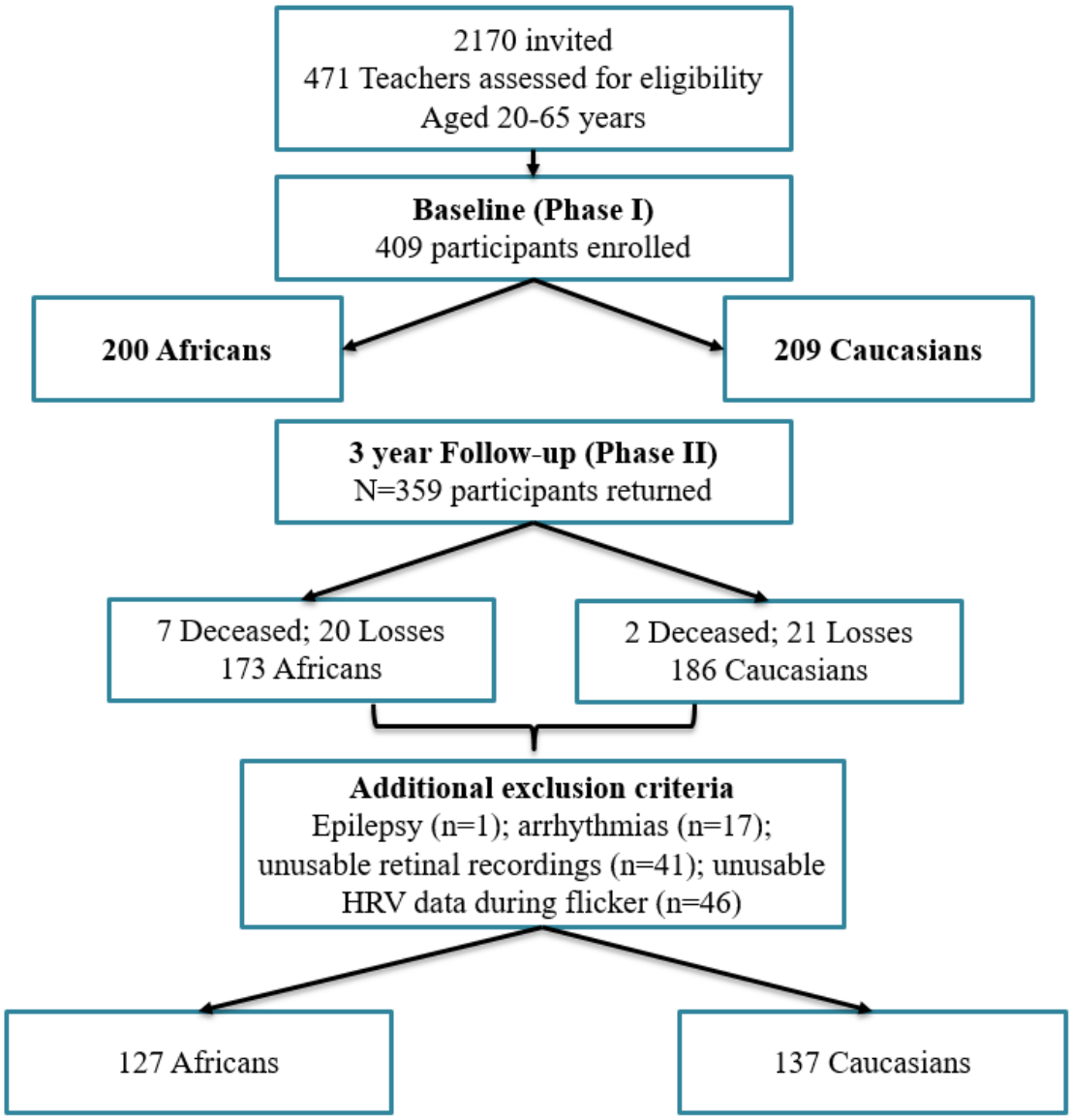

Figure 3.1: The Sympathetic activity and Ambulatory Blood Pressure in African (SABPA) prospective cohort stratified ethnic groups

Ethical considerations

The SABPA study obtained ethical approval from the Health Research Ethics Committee of the North-West University (NWU-000-360-7-S6). Written informed consent was obtained from all volunteers prior to participation. All procedures and objectives were explained to the participants prior to their recruitment, and the institutional guidelines, as stated by the Declaration of Helsinki (2004), were adhered to.

General procedure of investigation

Clinical assessments were done over a two-day period during the working week. Before 08h00 of the first clinical assessment day, four participating teachers were each fitted with an ambulatory blood pressure measurement (ABPM) and 2-lead ECG monitor device (Cardiotens CE120®; Meditech CE120; Meditech, Budapest, Hungary). A 24-hour standardized diet commenced and participants subsequently continued with their normal daily activities, reporting any peculiarities such as nausea, headaches, visual disturbances, palpitations, fainting, stress and physical activity, on the issued 24-hour diary cards. At 15h00, participants were transported to the NWU Metabolic Unit Research Facility for clinical measurements including the retinal vessel imaging. The Cardiotens® obtained HRV during retinal vessel analyses as well as event BP prior to and after retinal vessel assessments.

Retinal vessel analyses

Participants abstained from caffeinated and alcoholic beverages, smoking, strenuous physical activity and food consumption for at least 1 hour and no more than 5 hours prior to the measurements. A registered nurse familiarized them with the experimental setup and examined them for acute angle anterior chamber glaucoma risk. Fifteen minutes prior to the measurement, a drop of Tropicamide (1% Alcon 1% tropicamide and 0.01% benzalkonium chloride (m/v)), induced mydriasis in the right eye. If the right eye was not suitable, the left eye was used. The retinal vessel analyser (Imedos Systems UG, Jena, Germany) was used for digital fundus imaging, with a Carl Zeiss FF450^{Plus} camera (Carl Zeiss, Meditech Jena, Germany), to perform dynamic and static retinal vessel analyses.

For dynamic vessel analysis a standard flicker protocol by IMEDOS Systems was used. During FLIP, the duration of the baseline was 50 seconds, followed by a 20-second flicker period and an 80-second recovery (also referred to as second baseline) period. There were 3 flicker cycles in total, lasting an added total of 350 seconds for the entire measurement. The camera was set at a 30°degree angle with the participant focusing on the tip of a fixation rod, and one arteriole and one venular segment (as long as possible) were primarily selected in the upper or lower temporal quadrant of the fundus image. The quality of the FLIP measurements for each participant was assessed subjectively using a

newly developed, previously described, scoring method and extensively described by Kotliar et al [19] and in the *Supplementary Methods (**Appendix A**)*.

Absolute vessel diameters (measured in standardized measuring units (MU)) were determined for each measurement. Each one was calculated individually as the median value over the last 30 seconds of the first baseline phase prior to FLIP. Parameters derived from the smoothed averaged curve during FLIP, used in the current study, included: 1) the percentage maximal dilation in response to FLIP (MD), 2) The percentage absolute maximal constriction after FLIP (MC) was the minimum value occurring after maximum FLIP-induced dilation and was expressed as a percentage from baseline.

Dynamic vessel analysis with flicker stimulation was performed first, followed by image capturing for static vessel analysis. In static vessel analysis we calculated the central retinal artery and vein equivalent (CRAE and CRVE respectively) and subsequently determined the arteriovenous ratio (AVR) as previously described in the SABPA-study [17]. The presence of retinopathy and AV-nicking was determined by a registered ophthalmologist from a colour retinal image (*Supplementary Methods*).

Heart-rate-variability calculations during FLIP

HRV measures, time and frequency domain analyses were obtained with the Cardiotens® (Meditech CE120®; Meditech, Budapest, Hungary) and calculated using the CardioVisions® (Meditech CE120®; Meditech, Budapest, Hungary) software. The FLIP response window was viewed for each participant and a minimum window of 15 minutes was selected. This window was selected to include FLIP and post-FLIP analyses and is referred to as dynamic vessel analyses (DVA)-FLIP. The software removed all arrhythmias and extra-ventricular beats and additional outliers were manually removed before frequency and time domain variables were calculated for the selected window (Please refer to *Figure S6*). Variables used included time domain parameters, standard deviation of the NN intervals (SDNN), the root-mean square of the standard deviations of successive RR-intervals (rMSSD) as well as the triangular index (HRVti). The HRVti is an index of the pulse variability based on a triangular interpolation method in the given time interval. The histogram assesses the relationship between the total number of RR intervals detected and the RR interval variation. The triangular HRV index considers the major peak of the histogram as a triangle with its baseline width corresponding with the amount of RR interval variability, its height corresponds with the most frequently observed duration of RR intervals, and its area corresponds to the total number of all RR intervals used to construct it (*Figure S7a and 7b*). NN-intervals refer to the intervals between **normal** R-R peaks. Artefacts may arise during a measurement due to arrhythmic events or sensor errors [5]. This may lead to abnormal R-R peaks, which may in turn lead to incorrect statistical calculations. To ensure reliability and validity of data, only normal R-R peaks are selected. Please

refer to the methods diagram on how the HRV parameters were calculated (*Figure S7*). In practice, however, RR-intervals and NN-intervals are synonymous. The use of "NN-intervals" is to emphasize that the normal R-R peaks were used. Frequency domain parameters included the normalized units (nu) of the low frequency bands (LFnu), high frequency bands (HFnu), and the LF/HF ratio. Frequency domain analyses, over shorter periods of time, are more often employed as parameters since these are better equipped to discriminate between the contributions of SNS and parasympathetic (PNS), as they manifest in two overlapping frequency bands. The time domain parameters selected (specifically SDNN and rMSSD) are also used to investigate recordings of short durations [5, 20]. It is important to note that components of HRV provide measurement of the degree of autonomic modulations rather that the level of ANS tone [5]. Therefore, for the sake of clarity, all references to time domain parameters will be described in terms of SNS *activity* and references to frequency domain parameters will indicate SNS *modulation*. Please refer to Tables 1.1 and 1.2 for an overview of each HRV parameter**.**

Biochemical analyses

A registered nurse obtained fasting blood samples from the antebrachial vein branches of each participant's dominant arm with a sterile winged infusion set. Blood samples were dealt with in accordance with the standardised protocol and all samples were frozen at -80˚C until analysed in duplicate. cTnT was measured via an electrochemiluminecence method on the Roche® e411 (Roche®, Basel, Switzerland). Inter-batch variability 4.6%; intra-batch variability 4.2%. Below detectable limit cTnT values (31.3% of all cTnT analyses, N=104), were logarithmically calculated following a previously developed method [21].

Statistical analyses

Statistica version 13.3 (TIBCO Software Inc., Palo Alto, USA, 2018) was used for data analyses. Normality of all variables was tested within ethnic groups. Logarithmically transformed γGT, cotinine and HbA_{1c} levels were used in multivariable correlation models. Characteristics between ethnic groups were calculated with *t*-tests. Chi-square (X^2) statistics were used to analyse proportions and prevalence data. *A priori* defined covariates included age, body surface area (BSA), cotinine, γGT, HbA_{1c}, total cholesterol/HDL cholesterol ratio, nocturnal dipping status and 24-hour pulse pressure [25]. Single two-way ANCOVAs determined ethnic x gender differences for all HRV and retinal vessel markers, independent of *a priori* selected co-variates. One-way ANCOVA was used to determine the least square mean difference in response markers between ethnic groups, independent of *a priori* selected covariates. Multivariate linear regression analyses were used to determine associations between DVA parameters and HRV markers in several models. The dependent variables included 1) structure: CRAE, CRVE, AVR and 2) reactivity/functionality during FLIP: arteriole

maximum constriction, maximum dilation and venular maximum dilation. Independent variables included DVA-FLIP HRV variables SDNN, rMSSD, HRVti, LF, HF, LF/HF ratio and cTnT along with the *a priori* selected covariates in all models. All HRV parameters were calculated in separate models to avoid co-linearity, and baseline HR was adjusted for. Additional adjustment was made for CRVE in the CRAE models and vice versa. For all the aforementioned analyses, significance was set at $p<0.05$ (two-tailed) and the F to enter was fixed at 2.5 in regression models.

Sensitivity analyses

Forward stepwise regression analyses, with the same set of covariates, were repeated in several models in both ethnic groups. Excluding participants with diabetes (n=78), on diabetic treatment (n=29) and those using any form of hypertensive treatment (n=76), did not influence the outcome.

Results

We applied a hypothesis-driven approach where higher sympathetic activation [26, 27] and stroke risk [13] were shown in the SABPA Africans. Hence we commenced with the current investigation to determine ethnic differences for HRV responses to FLIP; Interaction testing revealed significant differences between Africans and Caucasians, but not between gender groups, independent of *a priori* selected covariates for CRVE ($F_{1, 254}$ = 8.0, p=0.003); Arteriole maximal dilation (%) ($F_{1, 240}$ = 19.27, p<0.001); Venular maximal dilation (%) ($F_{1, 240}$ = 20.65, p=0.024); cTnT ($F_{1, 254}$ = 27.1, p<0.001); FLIP rMSSD ($F_{1, 236}$ = 17.23, p<0.001); and FLIP HRVti ($F_{1, 236}$ = 5.30, p=0.003).

Baseline characteristics

Caucasians were older, Africans had lower BSA, but a poorer cardio-metabolic profile with higher CRP, HbA_{1c} and total cholesterol:HDL cholesterol levels than Caucasians (p<0.05). Similar cTnT values were observed between ethnic groups. Africans had higher 24-hour BP and PP, pre- and post-FLIP BP (p<0.001), IOP (p<0.001) and DOPP (p=0.010) values. Africans also showed a greater decrease in 24-hour HRV in both time and-frequency domain parameters, than did their Caucasian counterparts. Hypertensive/diabetic retinopathy (83% vs. 42%) and AV-nicking (77% vs. 26%) were more prevalent in Africans than in Caucasians (**Table 3.1**).

Adjusted retinal calibres and FLIP HRV parameters

In **Table 3.2**, the Africans revealed wider venules (p<0.001) and a smaller ARV (p<0.001). A comparison between dynamic retinal vessel parameters revealed that both arteriolar and venular maximum dilation in response to FLIP were greater in Africans (p<0.05). Lower SDNN, rMSSD and HRVti values during FLIP were evident in the African group (p=0.004). Frequency domain parameters during FLIP did not differ significantly between ethnicities. Overall, Africans presented with greater decreases in 24-hour and DVA-FLIP HRV than did Caucasians (**Figure 3.2**).

HRV, dynamic retinal calibres and SNS activity

During FLIP (**Table 3.3.1**), arteriolar maximum dilation inversely associated with rMSSD (*p*=0.030) in Africans. Again in this group, arteriolar maximum constriction inversely associated with both LFnu (p=0.003) and HFnu (p=0.021). Venular maximum dilation inversely associated with both SDNN and rMSSD, but positively with LF/HF (p<0.01) in Africans. However, in Caucasians, arteriolar maximum dilation positively associated with LFnu (p=0.004) and arteriolar maximum constriction positively associated with HRVti (p=0.015).

HRV, static retinal calibres and SNS activity

In (**Table 3.3.2**), inverse associations emerged for CRVE with SDNN and LFnu ($p=0.009$), as well as for AVR with rMSSD and HFnu ($p=0.027$), in Africans exclusively. In Caucasians there were positive associations of CRAE with HFnu, LF/HF, SDNN, rMSSD and HRVti ($p<0.05$). In contrast CRAE and AVR showed inverse associations with LFnu ($p=0.020$). In the same group, AVR was positively associated with HFnu, LF/HF, SDNN, rMSSD and HRVti ($p<0.05$). Additionally, systemic cTnT levels were inversely associated with rMSSD ($p=0.040$) in Africans only (**Table 3.3.3**).

Table 3.1: Baseline characteristics between ethnicities

Variable	Africans (N=127)	Caucasians (N=137)	*p*-value
Lifestyle and Biochemical measurements			
Age, years	47 ± 8	49 ± 9	0.011
Gender	69 (male)	61 (male)	
	58 (female)	76 (female)	
BSA (m^2)	1.94 ± 0.24	2.04 ± 0.29	0.001
Physical activity (kcal/day)	3318.47 ± 1257.10	3462.78 ± 1633.80	0.356
*Serum cotinine (ng/ml)	32.61 (18.60, 36.00)	21.63 (10.45, 31.00)	0.242
*γGT (U/L)	36.90 (22.00 ,62.80)	16.80 (10.60, 24.90)	<0.001
*CRP (mg/L)	4.31 (1.76, 7.82)	1.23 (0.58, 2.47)	<0.001
$HbA1_c$ (%)	6.16 ± 1.25	5.59 ± 0.69	<0.001
Total cholesterol: HDL	4.94 ± 1.68	4.29 ± 1.49	<0.001
cTnT (pg/mL)	4.58 ± 3.30	4.93 ± 3.36	0.364
Cardiovascular measurements			
24H ABPM SBP (mmHg)	139 ± 18	128 ± 11	<0.001
24H ABPM DBP (mmHg)	88 ± 11	79 ± 8	<0.001
24H ABPM PP (mmHg)	52 ± 10	48 ± 7	<0.001
DVA pre-FLIP SBP (mmHg)	141 ± 21	133 ± 15	<0.001
DVA pre-FLIP DBP (mmHg)	88 ± 13	83 ± 11	<0.001
DVA post-FLIP SBP (mmHg)	139 ± 19	132 ± 13	<0.001
DVA post-FLIP DBP (mmHg)	88 ± 13	83 ± 10	<0.001
IOP (R) (mmHg)	16.51 ± 4.14	14.95 ± 3.42	<0.001
DOPP (R) (mmHg)	71.68 ± 13.31	68.12 ± 10.77	0.007
24H HRV parameters			
Time domain parameters			
SDNN (ms)	106.10 ± 33.46	137.06 ± 37.47	<0.001
rMSSD (ms)	30.15 ± 14.26	31.08± 13.85	0.563

HRVti (N)	30.24 ± 10.37	37.23 ± 11.77	<0.001
Frequency domain parameters			
*LF (ms^2)	720.90 (360.00, 1003.00)	1009.79 (478.00, 1319,00)	<0.001
LFnu	65.64 ± 12.70	73.99 ± 10.39	<0.001
*HF (ms^2)	397.07 (138.00, 501.00)	390.88 (143.00, 456.00)	<0.001
HFnu	31.54 ± 11.35	25.27 ± 9.30	<0.001
*LF/HF	2.58 ± 1.69	3.44 ± 1.74	<0.001
Cardiovascular profile			
MI events, N (%)	1 (0.78)	1 (0.73)	0.959
Hypertensive, N (%)	104 (81.89)	45 (32.85)	<0.001
Diabetic, N (%)	97 (76.38)	29 (21.17)	0.003
Atrial fibrillation, N (%)	3 (2.63)	1 (0.73)	0.353
Hypertensive/ Diabetic Retinopathy, N (%)	106 (83.46)	57 (41.61)	<0.001
Arteriovenous nicking, N (%)	96 (76.90)	35 (25.54)	<0.001
Nocturnal dipping, N (%)	80 (63.00)	97 (70.80)	<0.001

*Data presented as median and interquartile ranges. Data expressed as arrhythmic mean ± SD. Where: N, number of participants; %, percentage change; CRP, C-reactive protein; HbA_{1c}, glycated haemoglobin; γGT, gamma glutamyl transferase; cTnT, cardiac Troponin T; ABPM, ambulatory blood pressure measurement; SBP, systolic blood pressure; DBP, diastolic blood pressure; IOP, intra-ocular perfusion pressure; DOPP, diastolic ocular perfusion pressure; FLIP, Flicker-light-induced-provocation; HRV, heart-rate-variability; SDNN, standard deviation of NN intervals; rMSSD, the square root of the men of the sum of the squares of differences between adjacent NN intervals; HRVti, heart rate variability triangular index; LFnu, power in low frequency range in normalized units; HFnu, power in high frequency range in normalized units; LF/HF, Ratio of LF(ms^2)/HF(ms^2); MI, myocardial infarction.

Table 3.2: Retinal Vessel and HRV profile of both ethnicities, independent of *a priori* co-variates

Variable	**Africans (N=127)**	**Caucasians (N=137)**	***p*-value**
Static retinal parameters			
CRAE (MU)	149.88 ± 1.31	151.01 ± 0.92	0.290
CRVE (MU)	249.20 ± 1.02	237.68 ± 1.60	<0.001
ARV	0.61 ± 0.005	0.64 ± 0.010	<0.001
Dynamic retinal parameters			
Artery MD (%)	3.99 ± 0.21	3.59 ± 0.18	0.029
Artery MC (%)	-1.49 ± 0.12	-1.73 ± 0.33	0.188
Vein MD (%)	4.72 ± 0.18	4.05 ± 0.20	0.002
HRV Time domain parameters during FLIP			
SDNN (ms)	75.91 ± 29.80	76.57 ± 18.36	0.004
rMSSD (ms)	24.52 ± 12.37	28.91 ± 68.06	0.004
HRVti (N)	16.22 ± 6.30	19.32 ± 6.89	<0.001
AUC data			
FLIP AUC_{artery}	55.49 ± 3.10	51.47 ± 2.70	0.111
Constriction AUC_{artery}	-10.68 ± 2.68	-20.22 ± 2.34	0.018
FLIP AUC_{vein}	58.02 ± 2.53	49.42 ± 2.28	0.027
HRV Frequency domain parameters during FLIP			
*LF (ms^2)	351.00 (192.00, 713.00)	340.00 (216.00, 710.00)	0.365
LFnu	59.08 ± 1.05	61.38 ± 1.54	0.414
*HF (ms^2)	195.00 (78.00, 538.00)	212.00 (96.00, 412.00)	0.782
HFnu	34.41 ± 1.30	34.13 ± 1.34	0.889
LF/HF	2.73 ± 0.25	2.48 ± 0.25	0.515
Biochemical marker			
cTnT (pg/mL)	4.80 ± 0.27	4.98 ± 0.25	0.344

*Data presented as median and interquartile ranges. Data expressed as arrhythmic mean ± SD. *A priori* covariates included age, gender, GGT, total cholesterol: HDL-cholesterol ratio, glycated

haemoglobin glucose (HbA_{1c}), hypertensive/ diabetic retinopathy, nocturnal dipping status and 24-hour pulse pressure. Where ARV, artery-to-vein ratio; FLIP, flicker-light-induced-provocation; AUC, area under the curve; ARV, arteriovenous ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; MC, maximum constriction; MD, maximum dilation; HRV, heart-rate-variability; SDNN, standard deviation of NN intervals; rMSSD, the square root of the men of the sum of the squares of differences between adjacent NN intervals; HRVti, heart rate variability triangular index; LFnu, power in low frequency range in normalized units; HFnu, power in high frequency range in normalized units; nu, normalised units; LF/HF, Ratio of LF(ms^2)/HF(ms^2).

Table 3.3.1: Independent associations between dynamic retinal vessel parameters and HRV measurements during FLIP between ethnicities.

Africans (N= 127)			
	Artery MD (%)	**Artery MC (%)**	**Vein MD (%)**
	ß (95% CI)	**ß (95% CI)**	**ß (95% CI)**
MODEL 1			
Adjusted R^2	**0.47**	**0.36**	**0.25**
Time domain			
SDNN (ms)	NS	NS	-0.29 (-0.51, -0.07) *p*=0.012
rMSSD (ms)	-0.37 (-0.68, -0.06) *p*=0.030	NS	-0.34 (-0.59, -0.10) *p*=0.002
HRVti (N)	NS	NS	NS
MODEL 2			
Adjusted R^2	**0.30**	**0.44**	**0.27**
Frequency domain			
LFnu	NS	-0.72 (-1.17, -0.27) *p*=0.003	NS
HFnu	NS	-0.53 (0.21) *p*=0.012	NS
LF/HF	NS	NS	0.42 (0.26, 0.58) *p*<0.001
Caucasians (N=137)			
	Artery MD (%)	**Artery MC (%)**	**Vein MD (%)**
	ß (95% CI)	**ß (95% CI)**	**ß (95% CI)**
MODEL 1			
Adjusted R^2	**0.14**	**0.32**	**NS**
Time domain			

SDNN (ms)	NS	NS	NS
rMSSD (ms)	NS	NS	NS
HRVti (N)	NS	0.53 (0.37, 0.68)	NS
		p=0.015	
		MODEL 2	
Adjusted R^2	**0.17**	**0.33**	**0.15**
Frequency domain			
LFnu	0.27 (0.09, 0.45)	NS	NS
	p=0.004		
HFnu	NS	NS	NS
LF/HF	NS	NS	NS

All analyses were adjusted for age, gender, GGT, total cholesterol: HDL-cholesterol ratio, glycated haemoglobin glucose (HbA_{1c}), 24-hour PP, hypertensive/ diabetic retinopathy and nocturnal dipping status. Abbreviations: ns, not significant; CI, confidence interval; MD, maximal dilation; MC, maximal constriction; SDNN, standard deviation of NN intervals; rMSSD, the square root of the men of the sum of the squares of differences between adjacent NN intervals; HRVti, heart-rate-variability triangular index; LF, power in low frequency range; HF, power in high frequency range; LF/HF, Ratio of LF(ms^2)/HF(ms^2).

Table 3.3.2: Independent associations between static retinal vessel parameters and HRV measurements during FLIP in both ethnicities.

Africans (N= 127)			
	CRAE	**CRVE**	**AVR**
	ß (95% CI)	**ß (95% CI)**	**ß (95% CI)**
MODEL 1			
Adjusted R^2	**0.28**	**0.22**	**0.18**
Frequency domain			
LFnu	NS	-0.38 (-0.65, -0.11) *p*=0.009	NS
HFnu	NS	NS	-0.34 (-0.63, -0.05) *p*=0.027
LF/HF	0.16 (0.003, 0.17) *p*=0.054	NS	NS
MODEL 2			
Adjusted R^2	**0.28**	**0.22**	**0.18**
Time domain			
SDNN (ms)	NS	-0.38 (-0.65, -0.11) *p*=0.009	NS
rMSSD (ms)	NS	NS	-0.34 (-0.63, -0.05) *p*=0.027
HRVti (N)	0.16 (0.003, 0.17) *p*=0.054	NS	NS
Caucasians (N=137)			
	CRAE	**CRVE**	**AVR**
	ß (95% CI)	**ß (95% CI)**	**ß (95% CI)**
MODEL 1			

Adjusted R^2	**0.45**	**-**	**0.15**
Frequency domain			
LFnu	-0.36 (-0.65, -0.07)	-	-0.52 (-0.89, -0.15)
	p=0.020		*p*=0.007
HFnu	0.57 (0.24, 0.90)	-	0.63 (0.20, 0.93)
	p=0.006		*p*=0.004
LF/HF	0.29 (0.07, 0.51)	-	0.31 (0.04, 0.58)
	p=0.055		*p*=0.031
MODEL 2			
Adjusted R^2	**0.45**	**-**	**0.15**
Time domain			
SDNN (ms)	0.36 (-0.65, -0.10)	-	0.52 (-0.89, -0.15)
	p=0.020		*p*=0.007
rMSSD (ms)	0.49 (0.16, 0.82)	-	0.63 (0.20, 0.98)
	p=0.006		*p*=0.004
HRVti (N)	0.22 (0.04, 0.44)	-	0.31 (0.04, 0.58)
	p=0.055		*p*=0.031

All analyses were adjusted for age, gender, GGT, total cholesterol: HDL-cholesterol ratio, glycated haemoglobin glucose (HbA_{1c}), 24-hour PP, hypertensive/ diabetic retinopathy, nocturnal dipping status. Additional adjustments were made for CRVE in the CRAE model and vice versa. Abbreviations: ns, not significant; CI, confidence interval; AVR, artery-to-vein ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; SDNN, standard deviation of NN intervals; rMSSD, the square root of the men of the sum of the squares of differences between adjacent NN intervals; HRVti, heart rate variability triangular index; LFnu, power in low frequency range in normalized units; HF, power in high frequency range in normalized units; LF/HF, Ratio of $LF(ms^2)/HF(ms^2)$.

Table 3.3.3: Independent associations between HRV measurements during FLIP and systemic levels of cTnT between ethnicities (*Supplementary Table*).

	Africans (N=127)			
	LFnu ß (95% CI)	**HFnu ß (95% CI)**	**SDNN ß (95% CI)**	**rMSSD ß (95% CI)**
Adjusted R^2	-	-	-	**0.14**
cTnT (ng/mL)	-	-	-	0.30 (0.02) *p*=0.040
	Caucasians (N=137)			
	LFnu ß (95% CI)	**HFnu ß (95% CI)**	**SDNN ß (95% CI)**	**rMSSD ß (95% CI)**
Adjusted R^2	-	-	-	**0.12**
cTnT (ng/mL)	-	-	-	-

All analyses were adjusted for age, gender, GGT, total cholesterol: HDL-cholesterol ratio, glycated haemoglobin glucose (HbA_{1c}), 24H PP, hypertensive/ diabetic retinopathy and nocturnal dipping status. Abbreviations: ns, not significant; CI, confidence interval; cTnT, cardiac Troponin T; SDNN, standard deviation of NN intervals; rMSSD, the square root of the men of the sum of the squares of differences between adjacent NN intervals; HRVti, heart rate variability triangular index; LF, power in low frequency range; HF, power in high frequency range.

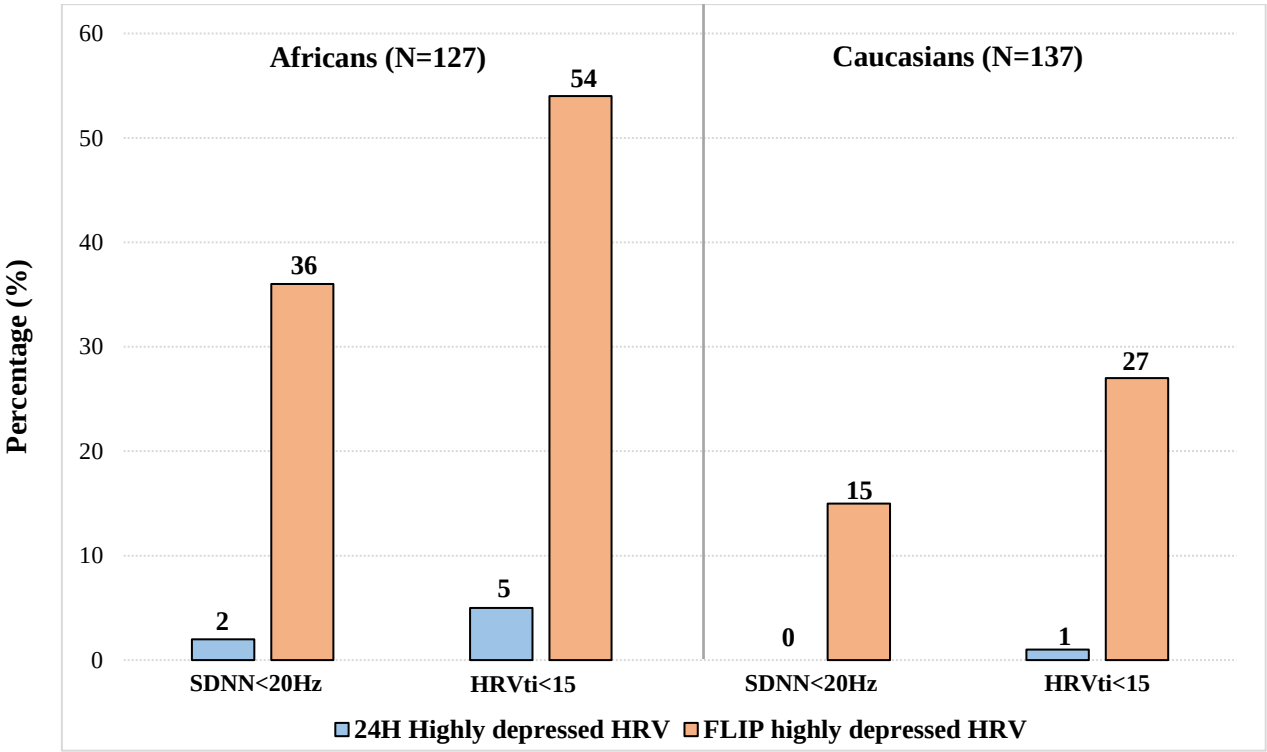

Figure 3.2: Comparison of highly depressed 24-hour (24H) and DVA-FLIP HRV parameters within ethnicities

Discussion

To our knowledge, this is the first study to link retinal structure and function with HRV parameters measured during DVA-FLIP. We investigated the relationship between retinal vessel structure and function, HRV parameters during DVA-FLIP analyses as well as the cardiac stress marker, cTnT, in a bi-ethnic cohort from South Africa. In Africans, increased arteriolar constriction and venular dilation to FLIP associated with decreased DVA-FLIP HRV (frequency- and time-domain measures). Higher systemic levels of cTnT were also associated with decreased HRV during FLIP. Findings support that dysregulation of the autonomic nervous system (ANS) may be associated with altered hemodynamics of the retinal and possibly the cerebral microvasculature. This may also imply that disrupted retinal (and cerebral) autoregulation may be a general result of systemic ANS dysfunction, indicating the importance of central control by the brain on microcirculatory systems in different vascular beds.

HRV, dynamic retinal calibres and SNS activity

Generally, myogenic, neurogenic and humoral processes are assumed to be involved in autoregulation of retinal vascular function [22]. Additionally, studies have indicated that retinal microvasculature reaction to increased demands depends on predisposing factors such as stress susceptibility, endothelial function, circulating neuroendocrine, cardiac peptides and inflammatory factors [23]. Several studies showed that the SABPA-Africans present with an altered stress responsivity, mainly related to SNS hyperactivity [9, 15, 17]. In the same cohort, such SNS hyperactivity associated with alterations in microvascular neural nitric oxide (NO) responses [14] and increased norepinephrine levels [17]. SNS hyperactivity is one of the main driving forces of hypertension [7]. Current findings support this notion as Africans presented with higher 24-hour BP, pre-and-post FLIP BP as well as higher pulse pressure. Such high-pressure conditions entail inadequate perfusion of peripheral tissues, but also diminished myogenic controlled mechanisms, defined as the micro-vessels' autoregulatory capacity [24, 25]. Autoregulation of the retinal microvasculature is apparently mainly mediated by responses to NO [28]. Interestingly, although retinal autoregulation was impaired in diabetic patients, retinal responses to exogenous NO were similar to those of healthy controls [29], implying maintained endothelium sensitivity to NO in diabetes. Therefore other factors, including ANS dysregulation and SNS hyperactivity, common in patients with diabetes, might play an important role in altered vaso-activity of retinal vessels.

Indeed, 37% of the African group were non-dippers – which in itself is an established feature of a sustained increase in SNS tone. Furthermore, 83% of the African cohort exhibited retinopathy that is prevalent in hypertension and diabetes, both of which relate to systemic autonomic neuropathy and increased SNS tone [30, 31]. Decreases in 24-hour ambulatory time (SDNN, rMSSD and HRVti) and frequency (LFnu, HFnu and LF/HF ratio) domain parameters were evident in Africans – more

so than in Caucasians. Reduced HRV indicates an increase in SNS activity and modulation and/or decreased vagal modulation [5]. A hyperactive SNS may compromise peripheral tissue perfusion, but also retinal and whole-brain perfusion [4, 8]. This indicates that FLIP may elicit ANS and particularly SNS-driven responses of the retinal vasculature and its autoregulative capacity.

In Africans attenuated retinal arteriolar, as well as venular dilatory responses, associated with decreased DVA-FLIP HRV time- and frequency-domain parameters. Arteriolar and venular dilation inversely associated with markers of PNS activity (rMSSD) and positively with increased SNS modulation (LFnu). This implies initial vagal withdrawal, possibly followed by increased SNS activity during DVA-FLIP. Long-term lower rMSSD is indicative of decreased vagal tone and has been linked to mental stress [5]. Such attenuated responses may reflect cerebral vasculature responses, and by recent investigations showing that vagal withdrawal relates to poorer whole-brain perfusion [4, 8]. Arteriolar constriction was inversely associated with both LFnu and HFnu, indicating a decreased power in all spectral bands, typically observed during and attributed to an insufficient SNS response [5]. Indeed, β-adrenergic hypo-responsivity, preceded by increased SNS activity, was reported in SABPA Africans [17]. In Africans the ability of the retinal vasculature to auto-regulate, decreases, as systemic autonomic dysfunction worsens. Preliminary histological evidence suggested the presence of alpha-adrenergic receptors in the mammalian retinal vessels [32], and the retinal vessels also contain catecholaminergic amacrine cells. Both infer that the ANS may directly influence the contractile abilities of the retinal microvasculature. Irrespective of these mechanisms, systemic hemodynamics may alter autoregulation capacity, depending on the degree of hemodynamic change that occurs prior to the microvasculature bed (Eularian conservation of Fluid) [33].Thus generalized SNS hyperactivity may elicit loss of tone and/or altered hemodynamics of the retinal vasculature. This also implies that if long-term SNS hyperactivity is present, impeded autoregulation should persist even after the stressor (FLIP) has ceased.

HRV, static retinal calibres and SNS activity

In Africans, wider venules and a smaller ARV were positively associated with increased SNS modulation (LFnu) and diminished vagal modulation (HFnu). This introduces the possibility that the FLIP-induced stress-response may persist due to the presence of general SNS hyperactivity. Therefore, sustained systemic SNS hyperactivity may govern a prolonged response to a stressor. SNS hyperactivity further encroaches on the retinal vasculature's autoregulatory capacities. In contrast, in Caucasians, post-FLIP static retinal analyses (arteriolar calibres and AVR) associated with both time- and frequency-domain parameters indicative of a restored ANS balance. Such a maintained sympatho-vagal balance is characterized by an increase in vagal modulation (HFnu) and decreased SNS modulation (LFnu) [5]. Our results support the notion that the SNS exerts a significant effect on smooth-muscle tone of the retinal vasculature, either directly or indirectly.

HRV during FLIP, cTnT and SNS activity – the brain-retina-heart link

We previously proposed a link between increased cTnT and attenuated retinal vasculature function [11]. Current findings support this, specifically within the context of cTnT's association with SNS function and FLIP as a mental/physical stressor. The inverse association between systemic cTnT and rMSSD during FLIP in Africans provides further support for the brain-heart link [33-35]. Higher levels of cTnT, as well as mental stress-induced increases in cTnT were related to SNS hyperactivity in Africans [9]. Furthermore, additional cTnT release was linked to increased activity of the midcingulate and subgenual anterior cingulate, located in the prefrontal cortex and identified as a primary seat of SNS activity [8]. This supports our observation of an association between cTnT and attenuated retinal arteriolar responses. The latter might link systemic levels of cTnT with vagal withdrawal and increased SNS tone.

Although speculative, we suggest that in the presence of pre-existing SNS hyperactivity, additional SNS activity provoked during FLIP further increases SNS modulation, thereby blood flow and activity in areas of the cingulate gyrus, possibly resulting in additional systemic cTnT release. Activation of the amygdala and the hippocampus also occur, and this cascade of activation finally launches a systemic response via input from the hypothalamus [7] and/or thalamus [36]. During FLIP an already hyperactive SNS will further be challenged, thereby causing further reduction in HRV indicating greater SNS activity and modulation, in an attempt to maintain adequate perfusion. Existing pressure-induced endothelial dysfunction and impaired smooth-muscle responses will manifest as attenuated retinal vessel dilatory and constriction responses. This increased SNS activity/modulation may lead to a prolonged vessel response, even after the stressor has ceased, indicating difficulty in re-establishing proper ANS balance. As a consequence the autoregulatory capacity of the retinal microvasculature will be impaired (**Figure 3.3**).

Translational relevance – From Cardiology to Ophthalmology

SNS hyperactivity may cause loss of tone and/or altered hemodynamics of the retinal microvasculature. Attenuated arteriolar and venular responses during FLIP, accompanied by decreased HRV variables, imply that the SNS exerts a significant effect on the smooth-muscle tone of the retinal vasculature, either directly or indirectly. Indeed, chemical stimulation of autonomic regulatory neurons in the dorsomedial and perifornical nuclei in the hypothalamus showed marked increases in IOP [37]. Agnifili et al [38] suggested that IOP variation was greater in patients suffering from primary open-angle glaucoma (POAG) than normal controls. Such fluctuations in IOP, together with altered hemodynamics and retinal blood-flow regulation, may possibly contribute to the pathogenesis of and glaucomatous damage in POAG [39]. The SABPA Africans with higher IOP and altered hemodynamics might thus be more susceptible to glaucomatous damage. Indeed, defective perfusion played a role in the appearance and exacerbation of glaucomatous optic

neuropathy [37, 39]. Sympathetic dominance and depressed HRV were also documented in patients with normal-tension glaucoma [37]. Additionally, associated vascular dysregulation was linked to SNS hyperactivity, manifested as blunted BP responses and decreased optic nerve head blood flow in response to cold provocation [40]. This might also indicate that the SABPA African cohort may be at a greater risk of possibly sustaining optic nerve and glaucotamous damage.

Limitations and recommendations

Limitations of the current study may include the specific population (only including African and Caucasian teachers from one demographic area) as well as the cross-sectional design, which prevents identification of physiological mechanistic cause-and-effect relationships. Several investigations have identified increased psychosocial stress experienced by this vulnerable African cohort, as one of the main contributors to this observed higher risk profile. However, the exploration of psycho-social stress influences are not within the scope of the current investigation and we suggest assessing the effect of psychosocial stress on the structure and function of the retinal vasculature, cardiac stress markers and stroke risk. We further recommend future retinal vessel investigations to include beat-to-beat BP monitoring, as cardio-metabolic demands increase during the FLIP, and blood pressure variations will occur in order to maintain homeostasis.

Conflict of interest

All authors declare no conflict of interest.

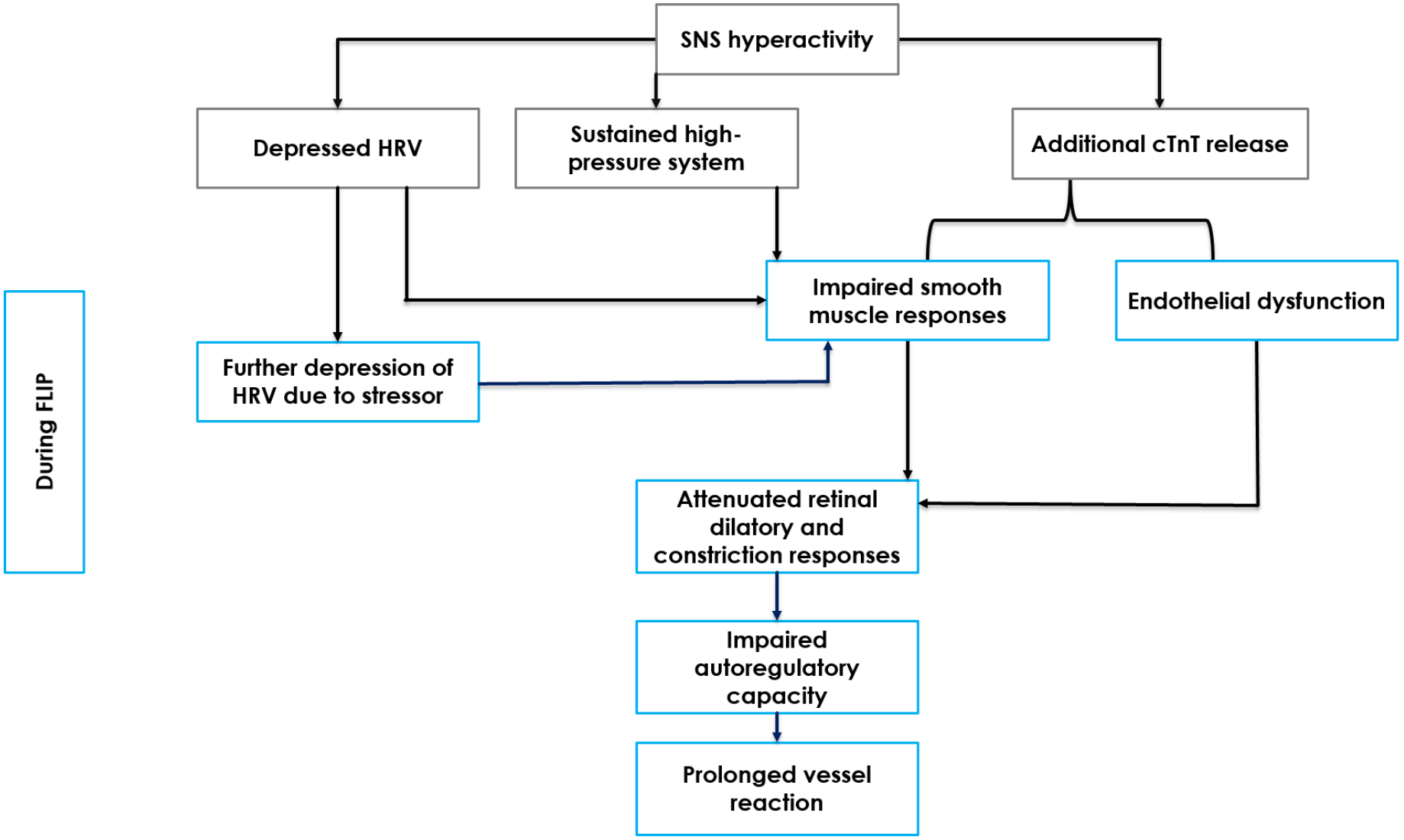

Figure 3.3: Possible series of events by which increased SNS tone may contribute to attenuated retinal vessel responses

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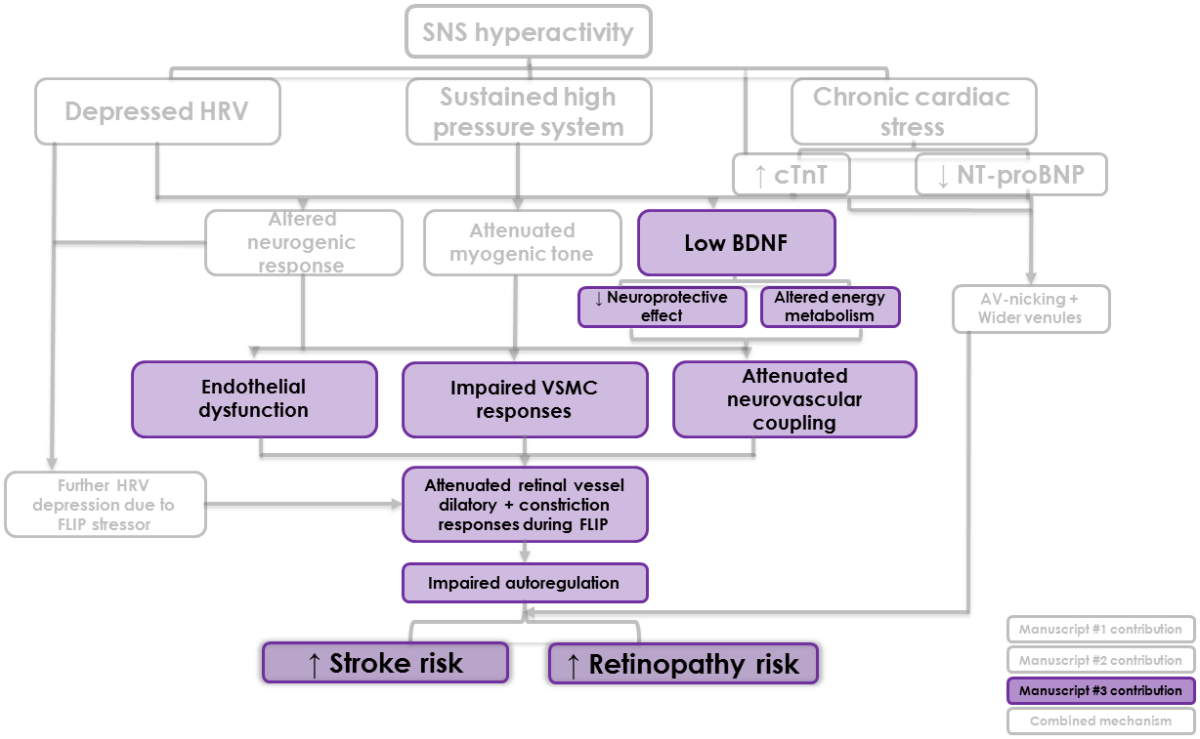
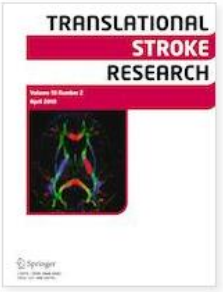
Chapter 4: Manuscript 3

Brain-derived-neurotrophic-factor reflects attenuated retinal vascular functionality and stroke risk: The SABPA study

As the previous two manuscripts supported a SNS-driven brain-retina-heart link, we aimed to further explore the central control factors that may influence neurovascular coupling and the retinal vasculature's autoregulatory abilities. Here we investigated associations between retinal arteriolar functionality during flicker-light-induced-provocation (FLIP) and systemic levels of brain-derived neurotrophic factor (BDNF) – the most abundant neurotrophin in the retina. We aimed to relate BDNF levels to potential stroke risk. Lower BDNF levels were observed in the total cohort compared to the normal reference ranges. However, low BDNF levels were only associated with attenuated arteriolar responses in the African group. Despite the greater 3-year increases in BDNF levels, the African group exclusively presented with attenuated arteriolar responses. Although low BDNF levels were also observed in Caucasians, BDNF's direct vasodilatory effect appeared to be more pronounced and protective in this group. Low BDNF levels predicted retinopathy and increased stroke risk, irrespective of race or gender. BDNF's direct action on vascular-smooth-muscle-cells may be impaired, and contribute to disturbed neurovascular coupling as well as increased stroke risk. This manuscript further exemplifies the brain-retina-heart link, again emphasizing how a hyperactive sympathetic nervous system may impact on the retinal autoregulatory processes.



This Manuscript has been presented at the 2019 ESM-EVBO Conference in Maastricht, The Netherlands, and has been submitted to the peer-reviewed journal *Translational Stroke Research* (Ref No TRSR-D-19-00144):

Journal Title	Translational Stroke Research 
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Low Brain-derived-neurotrophic-factor reflects attenuated retinal vascular function and increased stroke risk: The SABPA study

Short title: BDNF, retinal vessel dynamics, stroke risk

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Abstract

Assessment of retinal vessel function provides an automated approach to non-invasively assess the condition of the cerebral microvasculature and susceptibility to stroke. Brain-derived neurotrophic factor (BDNF) may constitute a potential link between cerebral and retinal blood flow, specifically alterations in local perfusion that occur in response to changes in neuronal activity, termed neurovascular coupling. We investigated associations between retinal vessel function during flicker-light-induced-provocation (FLIP) and systemic BDNF levels. A bi-ethnic cohort of teachers (N=280) aged 23-68 years was investigated. Prospective observations for serum BDNF and 24-hour blood pressure (BP) were obtained. During the 3-year follow-up measurements, retinal vessel calibres were quantified from mydriatic eye fundus images, and dynamic retinal vessel responses were determined during FLIP. The University of California stroke risk score was applied to assess subclinical 10-year stroke risk. Lower baseline and follow-up BDNF levels (1.3-1.8ng/mL) were observed in the total cohort compared to the normal reference ranges (6.97-42.6ng/mL). Compared to Caucasians greater, 3-year increases in BP and BDNF levels and a stronger prevalence of retinopathy at follow-up (73% vs 40%) were recorded in Africans. In Africans, arteriolar maximal dilation and maximal constriction was positively associated with follow-up BDNF (p=0.051 and p=0.029). Arteriolar diameter after flicker cessation was positively associated with follow-up BDNF (p<0.001). Arteriolar constriction time was inversely associated with follow-up BDNF in the Africans (p=0.035), yet positively in the Caucasians (p=0.024). BDNF changes (%) over 3 years were inversely associated with arteriolar dilation (p=0.032) and arteriolar constriction time (p=0.026), exclusively in the African group. Irrespective of race or gender, a novel BDNF cut-point of 1.5 ng/mL predicted the increased probability of hypertensive/diabetic retinopathy [AUC 0.60 (95% CI 0.45, 0.71); sens/spec 49%/68%), p=0.004]. The BDNF cut-point was associated with an increased 10-year stroke risk with an odds ratio of 1.56 (95% CI, 0.94; 2.06, p=0.011). The observed attenuation in retinal vessel responses and increased stroke risk may indicate a diminished neuro-protective effect of BDNF in the SABPA cohort. BDNF may possibly directly act on vascular-smooth-muscle-cells to alter arteriolar vascular resistance and contribute to disturbed neurovascular coupling and increased stroke risk.

Key words: BDNF; Retinal vessel dynamics; Flicker-light-induced-provocation; Stroke risk; Ethnicity; South Africa

Introduction

The retinal and cerebral microvasculature share many physiological and morphological characteristics [1, 2]. Analysis of the retinal vessels' structure and function further provides an automated and objective approach so as to non-invasively assess the cerebral microvasculature to possibly identify vascular pathologies [3] and stroke [4]. In a recent study, attenuated retinal arteriolar dilation responses during flicker-light-induced-provocation (FLIP) were related to increased stroke risk [5]. Such pathological conditions may manifest and/or present as a result of 1) disrupted autoregulation, usually the result of a sustained high-pressure system, leading to an eventual diminishing of myogenic control mechanisms (Bayliss effect), ultimately responsible for autoregulation [1, 6]; and 2) disturbed neurovascular coupling, the relationship between neural activity and blood flow [7]. Indeed, neurons, glial cells and the cerebral endothelium function as a cohesive unit, similar to that of the retinal microvasculature [2, 8, 9].

Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the retina (inner and outer retinal as well as the ganglion cell layer) [10, 11]. BDNF plays a key role in synaptic communication and plasticity [7], and is also associated with good neurovascular health – the local perfusion that adjusts in response to changes in neuronal activity [12]. BDNF is partly regulated by neuronal activity-dependent mechanisms such as during sensory stimulation and problem solving [11, 13], thereby implicating a link between maintaining cerebral and retinal blood flow during conditions of increased metabolic demand. Low BDNF levels have been linked to retinopathy [11, 14], increased risk of ischemic stroke [15], depression [13], anxiety, SNS activity and psychological stress [16, 17]. Recent evidence linked lower BDNF levels with structural endothelial dysfunction in Africans, but not in Caucasians [17]. It is important to note that BDNF levels measured in serum reflect brain-tissue BDNF levels across species [18].

To our knowledge, no study to date has examined systemic levels of BDNF in relation to retinal vasculature function during FLIP. Due to the well-documented stroke risk and attenuated arteriolar function in response to FLIP, previously shown in the SABPA African group [5, 19], we hypothesised that low systemic levels of BDNF will be associated with attenuated retinal arteriolar dilation and constriction during FLIP. Additionally, we aimed to examine stroke risk based on low BDNF levels, in accordance with ethnicity, within a South African cohort.

Materials and Methods

Study design and participants

The participants were initially recruited as part of phase 1 of the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study, well-described elsewhere [20]. All participants included in phase one (2008-2009) were invited to take part in the follow-up or phase two. Of the initial 409 participants, 359 reported for the second phase of the study. Only participants who took part in the second phase of the study, were included, as no retinal measurements were taken during the first phase. The study sample comprised urban male and female African and Caucasian teachers (N=359) from the North West Province, South Africa, aged between 23 and 68 years. The rationale for exclusively selecting teachers was to obtain a socio-economic equated sample from a similar working environment; cultural differences could however not be excluded. Additional exclusion criteria for this study included epilepsy (n=1), arrhythmias (n=17), unsuccessful retinal vessel recordings (n=41), incomplete dynamic vessel data (n= 20) and incomplete BDNF data (n= 7). Regarding retinal vessel reactivity measurements in response to FLIP, only individuals with a quality score of equal or greater than 2.5 were included (*Supplementary methods*). Finally, a total of 121 Africans and 159 Caucasians were included in the current study (sub-population N=280).

Ethical considerations

The SABPA study obtained ethical approval from the Health Research Ethics Committee of the North-West University (NWU-000-360-7-S6). Written informed consent was obtained from all volunteers prior to participation. All procedures and objectives were explained to the participants prior to recruitment and adhered to the institutional guidelines, as stated by the Declaration of Helsinki (2004).

General procedure of investigation

Clinical assessments were done over a two-day period during the working week. Before 08h00 of the first assessment day, four teachers were each fitted with an ambulatory blood pressure (ABPM) and 2-lead ECG monitor device (Cardiotens CE120®; Meditech CE120; Meditech, Budapest, Hungary). A 24-hour standardized diet commenced and participants subsequently continued with their normal daily activities, reporting any peculiarities such as nausea, headaches, visual disturbances, palpitations, fainting, stress and physical activity on a 24-hour diary card. At 15h00, participants were transported to the NWU Metabolic Unit Research Facility for clinical measurements, including retinal vessel imaging. The Cardiotens® obtained event BP prior and after retinal vessel assessments.

Retinal vessel analyses

Participants abstained from caffeinated and alcoholic beverages, smoking, strenuous physical activity and food consumption for at least 1 hour and no more than 5 hours prior to the measurements. They were familiarized with the experimental setup and were examined for acute angle anterior chamber glaucoma risk, with a pen light from the side of the eye by a registered nurse. Fifteen minutes prior to the measurement, a drop of Tropicamide (1% Alcon 1% tropicamide and 0.01% benzalkonium chloride (m/v)) was applied to induce mydriasis in the right eye. If the right eye was not suitable, the left eye was used. The retinal vessel analyser (Imedos Systems UG, Jena, Germany) was used for digital fundus imaging with a Carl Zeiss FF450^{Plus} camera (Carl Zeiss, Meditech Jena, Germany) to perform dynamic and static retinal vessel analyses.

For dynamic vessel analysis a standard flicker protocol by IMEDOS Systems was used. During FLIP, the duration of the baseline was 50 seconds, followed by a 20 second flicker period and an 80 second recovery (also referred to as second baseline) period. There were 3 flicker cycles in total, lasting an added total of 350 seconds for the entire measurement. The camera was set at a 30°degree angle with the participant focusing on the tip of a fixation rod, and one arteriole and one venular segment (the longest possible) were primarily selected in the upper or lower temporal quadrant of the fundus image. The quality of the FLIP measurements for each participant was assessed by experienced investigators using a newly developed, previously described, scoring method and extensively described in the *Supplementary Methods* (Appendix A). All additional methods such as supplementary heart-reate-variability calculations, are described in the *Supplementary Methods* (Appendix A).

Absolute vessel diameters (measured in standardized measuring units (MU)) were determined for each measurement. Each parameter was calculated individually as the median value over the last 30 seconds of the first baseline phase prior to FLIP. For the current study, parameters derived from the smoothed averaged curve during FLIP included: 1) the percentage maximal dilation in response to FLIP, 2) The percentage absolute maximal constriction after FLIP, referring to the minimum value occurring after maximum FLIP induced dilation and expressed as a percentage from baseline (*Figure S4*). The presence of retinopathy was determined by a registered ophthalmologist from colour retinal images.

Biochemical analyses

Fasting blood samples were obtained from the antebrachial vein branches of each participant's dominant arm with a sterile winged infusion set, by a registered nurse. Blood samples were handled according to the standardised protocol and serum and plasma samples were frozen at -80˚C until analysed in duplicates. Serum BDNF in ng/mL was determined via a quantikine colorometric-

sandwich immunoassay from R&D systems, Minneapolis, MN, USA. Samples were allowed to clot for 30min in a serum separator tube before centrifugation. The intra-assay and inter-assay precision were 3.8-6.2% and 7.6-11.3% respectively.

Stroke-risk markers

The 10-year UCLA risk composite score included gender, SBP, hypertensive drugs, diabetes, smoking habit, perfusion deficits, atrial fibrillation and electrocardiography (ECG) left ventricular hypertrophy (American Heart and Stroke certified UCLA Medical Centre, Primary Stroke Centre, Santa Monica, Los Angeles, USA). Medium to high probability of stroke was assumed based on scores of 5.2 and greater according to this risk score [5].

Statistical analyses

Statistica version 13.3 (TIBCO Software Inc., Palo Alto, USA, 2018) was used for data analyses. Normality of all variables was tested and γGT and HbA_{1c} were logarithmically transformed. Additionally, normality of retinal parameters were tested within ethnic groups, and arteriolar maximal dilation and arteriolar time to constrict were logarithmically transformed and used in regression models. Characteristics between ethnic groups were calculated with *t*-tests. Chi-square (X^2) statistics were used to analyse proportions and prevalence data. *A priori* defined covariates included age, gender, GGT, glycated haemoglobin glucose (HbA_{1c}), tumour necrosis factor-alpha, total cholesterol: HDL cholesterol ratio, diastolic ocular perfusion pressure and hypertensive/diabetic retinopathy. Single two-way ANCOVAs determined ethnic x gender differences for BDNF and retinal vessel markers, independent of *a priori* selected co-variates. One-way ANCOVA was used to determine the least square mean difference in response markers between ethnic groups, independent of *a priori* selected covariates. Multivariate linear regression analyses were used to determine associations between DVA parameters and BDNF in several models. The dependent variables included reactivity/function during FLIP: arteriole maximum constriction, maximum dilation, time to dilate, time to constrict and artery post-FLIP value, which is the arteriolar diameter measured 50-80s after flicker light cessation. Independent variables in separate models included baseline BDNF, follow-up BDNF, Δ% (changes in) BDNF over 3 year, along with the *a priori* selected covariates in all models. Percentage changes were calculated as follows: [(follow-up BDNF-baseline BDNF/baseline BDNF) X 100]. All BDNF models were computed separately as to avoid collinearity. For all the aforementioned analyses, significance was set at $p<0.05$ (two-tailed) and the F to enter was fixed at 2.5 in regression models.

Non-parametric receiver-operating-characteristics (ROC) analyses were performed to determine optimal BDNF cut-points predicting retinopathy for the maximum of the Youden index (J) (sensitivity + specificity – 1). Odds ratios (OR) (95% confidence intervals (CI)), were computed to

determine the probability of low BDNF levels to predict stroke risk. The probability of BDNF levels below 1.5ng/mL to predict medium-to-high UCLA 10-year stroke risk was thus calculated.

Sensitivity analyses

Forward stepwise regression analyses, with the same set of covariates were repeated in several models in both ethnic groups. Excluding participants using any form of hypertensive treatment (n=81), did not influence the outcome.

Results

We applied a hypothesis-driven approach where higher sympathetic activation (19, 20) and stroke risk (5) were shown in the SABPA Africans. Hence we commenced with the current investigation to determine ethnic differences, independent of *a priori* covariates, for BDNF ($F_{1, 282} = 11.07$, $p<0.001$), arteriole maximal dilation ($F_{1, 240} = 19.27$, $p<0.001$) and arteriolar time to constrict (s) ($F_{1, 240} = 20.65$, $p=0.024$).

Baseline characteristics

Caucasians were slightly older than Africans. Africans had lower body surface area with a poorer cardio-metabolic profile evidenced by higher HbA_{1c} and total cholesterol/HDL cholesterol ratio compared to their Caucasian counterparts ($p<0.05$). BDNF values did not significantly differ between ethnic groups. Africans showed higher pressure values [24h BP and PP, pre-and-post FLIP BP ($p<0.001$), intra-ocular pressure ($p<0.001$) and diastolic ocular perfusion pressure ($p=0.010$)]. Over 3 years Africans also showed an increase in BDNF levels compared to their Caucasian counterparts, who displayed a decrease in BDNF. Wider venules were also evident in the African vs the Caucasians group ($p<0.001$). Hypertensive/diabetic retinopathy (73% vs. 40%) was more prevalent in Africans than in Caucasians (**Table 4.1**).

Adjusted retinal vessel responses to FLIP

In **Table 4.2**, a comparison between dynamic retinal vessel parameters revealed that arteriolar maximal dilation in response to FLIP was greater in Africans ($p<0.05$). The time it took the arteriole to reach its maximum constriction was also prolonged in Africans compared to Caucasians ($p<0.05$).

Dynamic retinal vessel responses to FLIP and follow-up BDNF

In **Table 4.3**, arteriolar maximal dilation and maximal constriction were both positively associated with BDNF ($p=0.051$ and $p=0.029$) in Africans. Again in this group, the arteriolar diameter after flicker cessation was positively associated with BDNF ($p<0.001$), compared to an inverse relationship in Caucasians ($p=0.034$). Arteriolar constriction time was also inversely associated with BDNF in Africans ($p=0.035$), yet positively in Caucasians ($p=0.024$). In Caucasians exclusively, the arteriolar maximal constriction was positively associated with HbA_{1c} ($p=0.032$).

Dynamic retinal vessel responses to FLIP and changes in BDNF levels over three-years

Inverse associations between arteriolar maximal dilation and arteriolar constriction time with changes in BDNF levels were exclusively observed in the African group ($p=0.032$ and $p=0.026$ respectively). Again, in Africans, arteriolar dilation time was positively associated with changes in BDNF levels ($p=0.040$).

Stroke and retinopathy risk

Figure 4.1 shows that irrespective of race and gender, a novel BDNF cut-point of 1.5 ng/mL predicted the presence of hypertensive/diabetic retinopathy [AUC 0.60 (95% CI 0.45, 0.71); sensitivity/specificity 49%/68%; *p*= 0.004). Independent of race and gender, lower BDNF levels predicted an increased 10-year stroke risk with an odds ratio of 1.56 [Nagelkerke R^2= 0.33 (95% CI, 0.94; 2.06), p=0.011]. **Figure 4.2** is a fundus image of a male SABPA-African participant, with BDNF levels of 1.51 ng/mL, a UCLA stroke-risk score of 19.5 (high risk) and signs of diabetic and hypertensive retinopathy.

Table 4.1: Baseline characteristics between ethnicities

Variable	Africans (N=121)	Caucasians (N=159)	*p*-value
Lifestyle and Biochemical measurements			
Age, years	47 ± 8	49 ± 9	0.011
Gender (male: female)	59: 42	70:79	0.330
BSA (m^2)	1.93 ± 0.24	2.02 ± 0.29	0.004
Physical activity (kcal/day)	3363.47 ± 1257.10	3414.78 ± 1633.80	0.758
*Serum cotinine (ng/ml)	32.77 (18.60, 36.00)	18.61 (10.45, 31.00)	0.111
*γGT (U/L)	36.90 (22.00 ,62.80)	16.80 (10.60, 24.90)	<0.001
TNF-alpha (pg/mL)	2.45 ± 2.1	2.86 ± 1.68	0.069
$HbA1_c$ (%)	6.16 ± 1.25	5.59 ± 0.69	<0.001
*%Δ$HbA1_c$ over 3-year period	1.42 (-2.8, 5.25)	0.93 (-2.79, 2.94)	0.629
*Insulin (µU/mL)	12.04 (7.33, 14.28)	9.83 (5.26, 12.36)	0.011
*%ΔInsulin over 3-year period	-8.24 (-45.65, 9.94)	-13.43 (-35.44, 1.26)	0.309
*HOMA IR	2.35 (1.62, 3.78)	1.53 (0.97, 2.47)	<0.001
*%ΔHOMA IR over 3-year period	-22.77 (-42.65, 12.37)	-33.60 (-55.98, -14.41)	<0.001
Total cholesterol: HDL	4.94 ± 1.68	4.29 ± 1.49	<0.001
*Baseline BDNF (ng/mL)	1.32 (0.93, 1.85)	1.68 (1.18, 1.86)	0.006
*Follow-up BDNF (ng/mL)	1.80 (1.41, 2.20)	1.34 (0.89, 2.29)	0.007
*Change in BDNF over time (ng/mL)	0.40 (-0.15, 0.93)	-0.12 (-0.59, 0.46)	<0.001
*Change in BDNF over time (%)	28 (-7.59, 75.22)	-7.80 (-36.94, 28.21)	<0.001
Cardiovascular measurements			
24H ABPM SBP (mmHg)	141 ± 18	128 ± 11	<0.001
24H ABPM DBP (mmHg)	89 ± 12	79 ± 7	<0.001
24H ABPM PP (mmHg)	53 ± 11	48 ± 8	<0.001
DVA pre-FLIP SBP (mmHg)	143 ± 19	134 ± 14	<0.001
DVA pre-FLIP DBP (mmHg)	88 ± 11	85 ± 11	<0.001

DVA post-FLIP SBP (mmHg)	139 ± 20	133 ± 15	<0.001
DVA post-FLIP DBP (mmHg)	88 ± 13	83 ± 10	<0.001
IOP (R) (mmHg)	18.70 ± 3.25	15.92 ± 3.44	<0.001
DOPP (R) (mmHg)	72.62 ± 14.32	68.18 ± 11.41	0.007
Static retinal vessel parameters			
CRAE (MU)	149.96 ± 1.20	151.01 ± 1.00	0.348
CRVE (MU)	249.92 ± 1.80	236.54 ± 1.60	<0.001
ARV	0.60 ± 0.06	0.64 ± 0.04	<0.001
Dynamic retinal vessel parameters			
*Arteriole maximum dilation (%)	4.15 (2.24, 5.50)	3.38 (1.96, 5.12)	0.065
Arteriole time to dilate (s)	17.66 ± 5.16	17.54 ± 5.06	0.855
Arteriole maximum constriction (%)	-1.52 ± 1.17	-1.74 ± 1.33	0.156
*Arteriole time to constrict (s)	51.00 (43.00, 66.00)	44.00 (37.00, 54.00)	<0.001
Arteriole post-FLIP value (%)	100.07 ± 0.74	100.35 ± 0.60	<0.001
Cardiovascular Profile			
MI events, N (%)	1 (0.78)	1 (0.73)	0.959
Hypertensive, N (%)	92 (76.03)	59 (37.12)	<0.001
Diabetic, N (%)	83 (68.60)	35 (22.01)	<0.001
Atrial fibrillation, N (%)	3 (2.48)	1 (0.63)	0.353
Hypertensive/ Diabetic Retinopathy, N (%)	89 (73.55)	65 (40.88)	0.004

*Data presented as median and interquartile ranges. Data expressed as arrhythmic mean ± SD. Where: N, number of participants; % percentage change; %Δ, percentage change over 3 years; TNF-alpha, tumour necrotic facto alpha; HbA_{1c}, glycated haemoglobin; γGT, gamma glutamyl transferase; BDNF, brain derived neurotrophic factor; ABPM, ambulatory blood pressure measurement; SBP, systolic blood pressure; DBP, diastolic blood pressure; IOP, intra-ocular perfusion pressure; DOPP, diastolic ocular perfusion pressure; FLIP, Flicker light induced provocation; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; AVR, arteriovenuos ratio; MI, myocardial infarction.

Table 4.2: Comparison of dynamic arteriolar responses during FLIP and systemic levels of BDNF between ethnicities, independent of *a priori* covariates

Variable	Africans (N=121)	Caucasians (N=159)	*p* value
Dynamic retinal arteriolar analyses			
Maximum dilation (%)	3.93 ± 0.22	3.61 ± 0.20	0.104
Time to maximal dilation (s)	17.86 ± 0.54	17.28 ± 0.50	0.976
Maximum constriction (%)	-1.55 ± 0.12	-1.70 ± 0.12	0.096
Time to maximal constriction (s)	53.24 ± 1.49	48.35 ± 1.34	<0.001
Post-FLIP value (%)	100.12 ± 0.07	100.37 ± 0.07	0.012
BDNF (ng/mL)	1.89 ± 0.07	1.70 ± 0.08	0.062

Data expressed as an arithmetic mean (± SE). *A priori* covariates included age, gender, GGT, tumour necrotic factor alpha, total cholesterol: HDL cholesterol ratio, diastolic ocular perfusion pressure and hypertensive/diabetic retinopathy. Abbreviations: ns, not significant; CI, confidence interval; FLIP, flicker light induced provocation; BDNF, brain derived neurotrophic factor.

Table 4.3: Independent associations between dynamic retinal arteriolar parameters and BDNF between ethnicities.

	Maximum dilation (%) ß (95% CI)	**Time to dilate (s) ß (95% CI)**	**Maximum constriction (%) ß (95% CI)**	**Time to Constrict (s) ß (95% CI)**	**Post-FLIP value (%) ß (95% CI)**
Africans (N= 121)					
Cross-sectional observations					
Adjusted R^2	**0.15**	**<0.10**	**<0.10**	**<0.10**	**<0.10**
Baseline BDNF (ng/mL)	NS	-	-	-	-
Adjusted R^2	**0.21**	**0.22**	**<0.10**	**0.12**	**0.48**
Follow-up BDNF (ng/mL)	0.28 (0.01, 0.55) *p* =0.051	NS	0.13 (0.08, 0.31) *p* =0.029	-0.14 (-0.29, -0.03) *p* =0.035	0.33 (0.15, 0.51) *p* <0.001
HbA_{1c}	NS	NS	NS	NS	NS
Prospective observations					
Adjusted R^2	**0.21**	**0.11**	**0.11**	**0.18**	**0.13**
%Δ BDNF levels over 3-years	-0.31 (-0.58, -0.04) *p* =0.032	0.20 (0.01, 0.40) *p* =0.040	NS	-0.15 (-0.33, -0.03) *p* =0.026	0.28 (0.19, 0.37) *p*=0.005
Caucasians (N=159)					

	Maximum dilation (%) ß (95% CI)	**Time to dilate (s) ß (95% CI)**	**Maximum constriction (%) ß (95% CI)**	**Time to Constrict (s) ß (95% CI)**	**Post-FLIP value (%) ß (95% CI)**
Cross-sectional observations					
Adjusted R^2	**0.13**	**<0.10**	**0.12**	**0.10**	**<0.10**
Baseline BDNF (ng/mL)	0.12 (0.07) *p* =0.10	-	-	NS	-
Adjusted R^2	**0.23**	**<0.10**	**0.18**	**<0.10**	**0.37**
Follow-up BDNF (ng/mL)	NS	NS	NS	0.10 (0.08, 0.28) *p* =0.024	-0.16 (-0.31, -0.03) *p* =0.033
HbA_{1c}	0.35 (0.23, 0.47) *p*=0.006	NS	0.16 (0.02, 0.30) *p* =0.032	NS	NS
Prospective observations					
Adjusted R^2	**0.15**	**<0.10**	**<0.10**	**0.13**	**<0.10**
%Δ BDNF levels over 3-years	-0.14 (-0.28, -0.004) *p* =0.064	NS	NS	NS	NS

All analyses were adjusted for age, gender, GGT, tumour necrotic factor alpha, total cholesterol: HDL cholesterol ratio, diastolic ocular perfusion pressure, and hypertensive/diabetic retinopathy. All BDNF models were separately calculated as to prevent co-linearly influenced results. Abbreviations: ns, not significant; CI, confidence interval; %Δ , percentage change; BDNF, brain derived neurotrophic factor; HbA_{1c}, glycated haemoglobin.

Table 4.4: Independent associations between Follow-up BDNF levels, HRV measurements during FLIP and intraocular pressure between ethnicities (*Supplementary Table*)

	Africans (N=121)			
	LFnu **ß (95% CI)**	**HFnu** **ß (95% CI)**	**HRVti** **ß (95% CI)**	**IOP (mmHg)** **ß (95% CI)**
Adjusted R^2	**0.46**	**0.32**	**0.10**	**0.26**
Follow-up BDNF (ng/mL)	-0.68 (-0.17) *p*=0.009	0.22 (0.09) *p*=0.013	-0.20 (0.09) *p*= 0.030	-0.30 (0.132) p=0.047
	Caucasians (N=159)			
	LFnu **ß (95% CI)**	**HFnu** **ß (95% CI)**	**HRVti** **ß (95% CI)**	**IOP (mmHg)** **ß (95% CI)**
Adjusted R^2	**-**	**-**	**-**	**<0.1**
Follow-up BDNF (ng/mL)	-	-	-	-

All analyses were adjusted for age, gender, GGT, total cholesterol: HDL-cholesterol ratio, glycated haemoglobin glucose (HbA_{1c}), 24H PP, hypertensive/ diabetic retinopathy. Abbreviations: ns, not significant; CI, confidence interval; BDNF, brain-derived neurotrophic factor; rMSSD, the square root of the mean of the sum of the squares of differences between adjacent NN intervals; HRVti, heart rate variability triangular index; LF, power in low frequency range; HF, power in high frequency range; IOP, intraocular pressure.

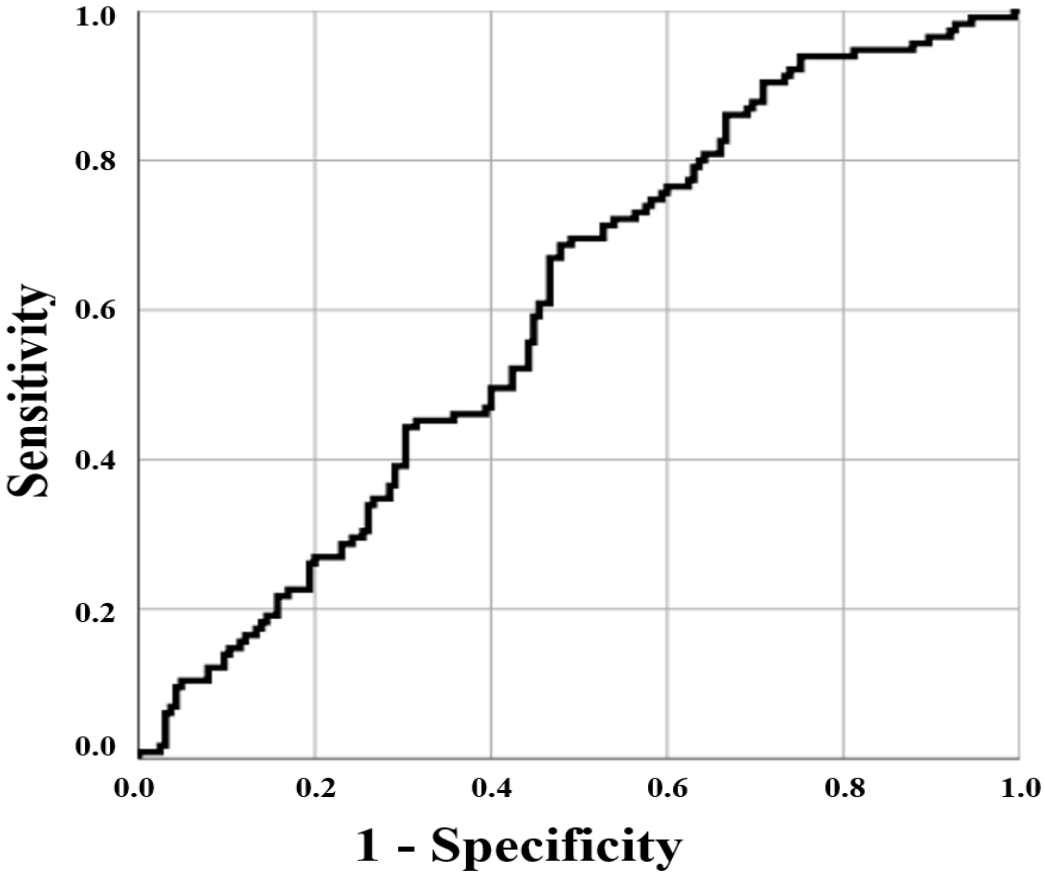

Figure 4.1: ROC curve depicting the serum BDNF cut-point for retinopathy in both Africans and Caucasians. AUC 0.60 (95% CI 0.45, 0.71; sensitivity/specificity 49%/68%; *p*=0.004). Where AUC, area under the curve; CI, confidence interval of 95%; BDNF, brain-derived neurotrophic factor.

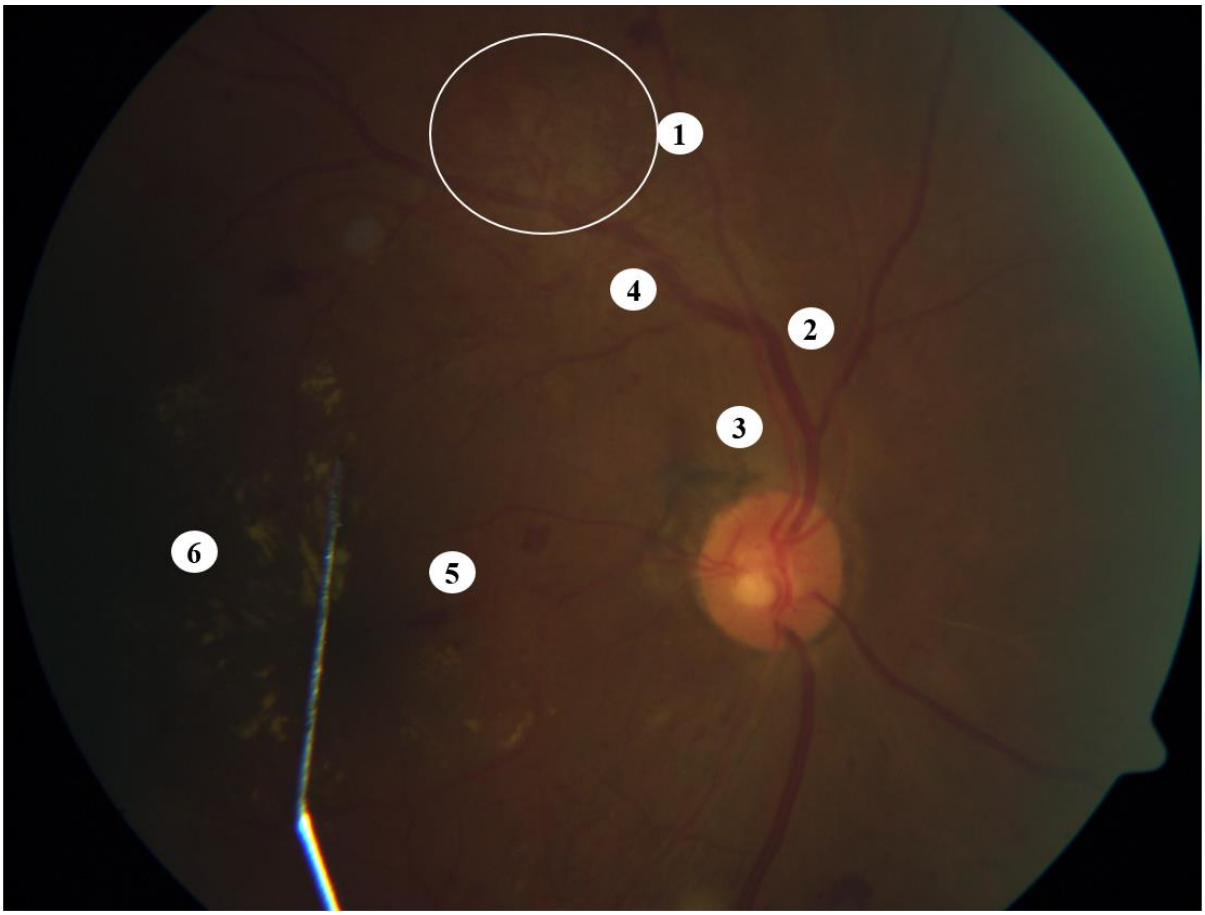

Figure 4.2: Retinal image of a male African participant with BDNF levels of 1.51ng/mL and a UCLA stroke risk score of 19.5 (high risk). Signs of retinopathy are 1) Neovascularisation, 2) Wider venules, 3) arteriolar narrowing, 4) AV-nicking, 5) Haemorrhages, 6) Hard exudates.

Discussion

To our knowledge, this is the first study to link retinal vessel function, during an acute stressor (FLIP) with systemic, serum levels of BDNF in a bi-ethnic cohort. Although both Africans and Caucasians presented with BDNF levels far below the reference range, attenuated retinal arteriolar responses due to FLIP was associated with BDNF in the African group exclusively. Our findings in Africans revealed that attenuated retinal arteriolar variables (maximal constriction, time to constrict and arteriolar diameter after flicker cessation) were associated with lower serum BDNF levels. Despite these attenuated responses being exclusively present in Africans, low BDNF levels predicted an increased stroke risk in both ethnicities. A novel BDNF cut-point of 1.5ng/mL predicted the development of retinopathy, again irrespective of ethnicity. The low BDNF levels observed in the SABPA cohort may indicate that BDNF's neuro-protective effect is attenuated in our cohort.

BDNF levels at follow-up, stroke risk and dynamic retinal arteriolar responses

BDNF has recently been identified as a key player in neurovascular coupling – the intrinsic relationship that exists between neural activity and subsequent changes in blood flow. This exemplifies its neuroprotective effect, specifically during circumstances of ischemia and hypoglycaemia, maintaining sufficient cerebral and, possibly, increased retinal blood flow during conditions of greater metabolic demand [12, 21]. Although both our African and Caucasian groups presented with BDNF levels below the reference ranges (1.3-18.ng/mL vs 6.97-42.6ng/mL) [17, 22], ethnic-specific associations existed between BDNF and arteriolar constriction responses during FLIP. In Africans, lower BDNF levels were associated with attenuated retinal arteriolar maximal constriction during FLIP, as well as delayed arteriolar constriction time. BDNF levels were also positively associated with arteriolar maximal dilation and the observed average arteriolar diameter after flicker cessation. The retinal arterioles showed greater dilation at lower BDNF levels, yet with a pronounced delay in their constriction ability and delayed reduction in their diameter after cessation of flicker-light. This finding is supported by Kotliar and co-workers, which reported a comparable arteriolar response in patients suffering from Alzheimer's disease – a disease where disrupted neurovascular coupling is evident [23]. It appears that, in Africans, the lower BDNF levels, might reflect disrupted neurovascular coupling. Dilation did occur, ensuring adequate blood supply, yet the counteracting constriction mechanisms displayed a delayed response [23, 24]. This may also indicate a prolonged retrograde propagation of the vascular response [25]. The greater stress susceptibility, moderate depression and compensatory sympathetic hyperactivity [26-29], reported in Africans, may result in lower BDNF levels and modified hemodynamics. Usually BDNF enhances SNS drive, but it is possible that sustained SNS hyperactivity may lead to the eventual diminishing or blunting of BDNF's actions – resulting in lower BDNF levels. This statement is supported by decreased heart rate variability, during FLIP, associating with decreased BDNF levels (**Table 4.4**). The latter may

hint at altered retinal vasculature smooth-muscle responses and endothelial dysfunction [17, 29]. Sustained SNS hyperactivity, resulting in a modified hemodynamic profile might contribute to diminished myogenic control mechanisms and disturbed autoregulation, evidenced by the attenuated arteriolar constriction responses and delayed diameter recovery.

Another possible explanation for the attenuated arteriolar responses in Africans may pertain to glial cell function. Retinal Müller cells are one of the main targets of BDNF [10, 30], and some findings suggest that Müller cells actively secrete BDNF during metabolically taxing situations [10]. Lower BDNF levels have indeed been linked to depressed heart-rate-variability and SNS dysregulation [21]. Again, BDNF levels measured in serum reflect brain-tissue BDNF levels across species [18]. In addition, higher systemic BP, perfusion pressure and accompanying IOP observed in the African cohort, may interrupt retrograde transport of BDNF to their targeted retinal ganglion and Müller cells [11, 31]. Indeed, lower BDNF levels were associated with increased IOP in this African cohort (**Table 4.4**). Lower BDNF levels have also been associated with apoptosis and neurodegeneration, which may eventually lead to neurovascular damage [7, 11, 12], consequently altering neurovascular coupling. Other factors such as increased IOP and related ischemia may alter BDNF receptor expression on Müller cells [30]. Furthermore, a failure in the BDNF supply to retinal ganglion cells leads to retinal ganglion cell death and subsequent retinopathy [11, 13].

Despite the lower BDNF levels in the Caucasian group, BDNF was not associated with attenuated arteriolar constriction time or the average arteriolar diameter after flicker cessation. However, their glycated haemoglobin (HbA_{1c}) was associated with attenuated arteriolar constriction. Besides BDNF's neuroprotective effect, it also plays a central role in energy homeostasis, and has a pronounced anti-diabetic effect [32, 33]. We cautiously suggest that, despite slightly lower BDNF levels, BDNF's direct vasodilatory effect may be more protective and pronounced in Caucasians. This notion possibly explains the relationship between BDNF and arteriolar dilation. However, it is possible that in Caucasians, lower BDNF levels may adversely affect peripheral glucose metabolism and tissue sensitivity for glucose, possibly supporting the relationship between HbA_{1c} and attenuated arteriolar constriction.

Low BDNF levels, stroke and retinopathy risk

The majority of our study participants presented with hypertensive/ diabetic retinopathy (55%), which is linked to increased stroke risk [34]. Despite the contrasting associations observed, lower BDNF levels predicted a 1.56-fold increased 10-year stroke risk independent of ethnicity and gender. Low BDNF levels have been associated with poorer long-term functional outcome following ischemic stroke [15]. Particularly ischemic stroke is characterised by early onset, excitotoxicity-induced neuronal injury [22]. This may exacerbate neuronal damage via apoptotic mechanisms, exemplifying the loss of BDNF's neuro-protective mechanisms. Furthermore, a novel BDNF cut-

point of 1.5ng/mL predicted the development of hypertensive/diabetic retinopathy, again irrespective of ethnicity and gender.

Changes in BDNF levels over three years and retinal arteriolar responses

Over the course of three years, BDNF levels increased in the African group, but decreased in the Caucasian group. The up-regulation of BDNF over three years in Africans was a surprising result. This might possibly reflect an attempt to maintain optimal neurovascular function; yet, when they are chronically challenged, BDNF's protective mechanisms may fail, despite compensatory attempts to increase BDNF's levels. We cautiously propose that this risk profile in the African group may mainly be driven by the loss of the neuroprotective influence and disrupted neurovascular coupling. This might also possibly be the result of sustained chronic stress, SNS hyperactivity, high-pressure systems (BP, IOP) and cardiac stress [5, 17, 19, 26-29, 35, 36]. This proposition is supported by the retinopathy profile observed in a male African participant with BDNF levels of 1.51 ng/mL and a high 10-year stroke-risk probability (**Figure 4.2**). This may also ultimately relate to the loss of myogenic control mechanisms, disrupted autoregulation and endothelial dysfunction, manifesting as attenuated retinal arteriolar responses to FLIP. In contrast, in the Caucasian group, factors essentially impacting on and contributing to glucose metabolism and acute stress [29] may increase their risk. Down-regulation in BDNF levels in Caucasians may thus possibly accompany a physiological down-regulation of insulin – although such a downregulation is not necessarily evident of insulin resistance (as reflected by the HOMA IR) [37]. We carefully suggest that the down-regulation of insulin accompanies a consequential increase in HbA_{1c}, as a possible metabolic compensatory mechanism, while not yet pathological. Indeed, although brain-insulin regulation is independent of systemic changes in blood-glucose levels [38], the retina is sensitive to systemic changes in insulin and glucose levels [39, 40]. Thus retinal arteriolar function may be affected by systemic insulin and glucose sensitivity.

Conclusions

The neuro-protective effect of BDNF might be attenuated in both SABPA Africans and Caucasians, reflecting an increased 10-year stroke risk as an implication. BDNF's direct action on vascular smooth-muscle cells might be modified with the altered arteriolar vascular resistance contributing to disturbed neurovascular coupling to an increased risk of retinopathy and stroke. The risk profile of Africans would seem to be mainly driven by the loss of neuroprotective influences, disrupted neurovascular coupling, SNS hyperactivity and higher circulatory pressure. In the Caucasian group, we suggest that this risk profile is mainly driven by factors essentially impacting on and contributing to glucose metabolism and acute stress (**Figure 4.3**).

Limitations and Recommendations

Limitations of the current study include the specific population of African and Caucasians teachers from one demographic area. As retinal vessel analyses were only completed during the follow-up phase of SABPA, cause-and-effect inferences cannot be made. Furthermore, as the risk profile differs distinctly between our African and Caucasian teachers, we were unable to evaluate pair matched risk-case, so as to determine whether the differences are ethnic specific, or whether the results were due to the observed higher cardiovascular risk in the African cohort. We recommend prospective analyses of additional glial cell and endothelial markers, to further elucidate the contribution of the neurovascular component to retinal vessel dynamics. Furthermore, analyses on the clearance and metabolism of BDNF should be conducted to more accurately describe possible mechanistic inferences.

Compliance with ethical standards

The SABPA study obtained ethical approval from the Health Research Ethics Committee (HREC) of the NWU and extended approval was granted for the second phase (Ethics number: **NWU-00036-07-S6)**. Written informed consent was obtained from all volunteers prior to participation. All procedures and objectives were explained to the participants prior to their recruitment, and adhered to the applicable institutional guidelines and terms, as stated by the Declaration of Helsinki (2004).

Conflict of interest

All authors declare no conflict of interest. Miss A Wentzel declares no conflict of interest, Prof L Malan declares no conflict of interest, Prof W Smith declares no conflict of interest, Prof Dr R von Känel declares no conflict of interest and Prof NT Malan declares no conflict of interest.

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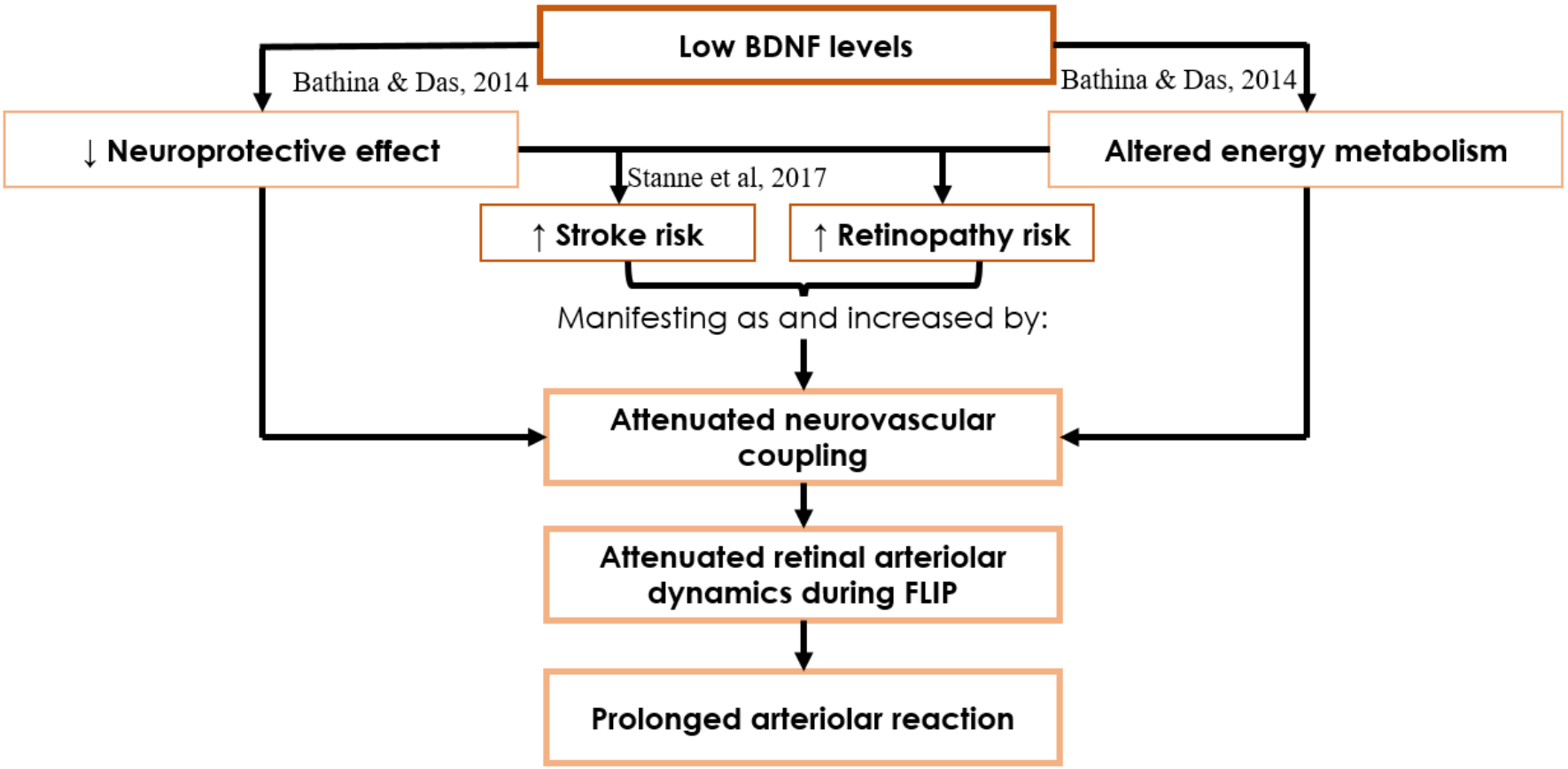

Figure 4.3: The proposed series of events possibly contributing to decreased BDNF levels, the attenuated retinal arteriolar responses observed and evident risk

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Chapter 5

General Findings and Conclusion

General Findings and Conclusions

1. Introduction

This chapter summarizes the main findings of the three manuscripts presented in this thesis. A thorough discussion and conclusions, elucidating the interpretation of all results and comparisons with the relevant literature, reviewed in *Chapter 1*, will follow. Additionally, limitations of the current study as well as recommendations for future research, specifically pertaining to retinal vessel analyses, cardiac stress, SNS analyses and neurotrophic factors, are proposed – specifically within the context of SNS hyperactivity.

The overall conclusion of the current study exemplifies the possible role of SNS hyperactivity in the control of retinal vascular dynamics. This role is emphasized as the SNS function is evident in each approach followed: SNS hyperactivity was associated with increased SNS-linked cardiac stress markers (cTnT and NT-proBNP) and may have indirectly contributed to the associations between retinal vascular dynamics and cTnT and NT-proBNP (influence on myogenic control mechanisms). Hence, in the second manuscript markers for SNS activity, including time-domain HRV (SDNN), was assessed. The association between SNS activity and modulation, and the retinal vessel dynamics during FLIP, reflected a direct/ indirect influence of the SNS on the retinal vasculature's function (neurogenic control and neurovascular coupling mechanisms). In the third and final manuscript, lower BDNF levels associated with attenuated retinal vessel responses. BDNF is linked to SNS activity, associated with neurovascular health, and identified as a key player in effective neurovascular coupling. Thus the lower BDNF levels and associated attenuated retinal arteriolar responses reflected a possible compromised neurovascular coupling, linked to stroke risk. These observations were carefully attributed to sustained SNS hyperactivity responsible for long-term high-pressure exposure, sustained cardiac stress and low BDNF levels. In all findings, the reoccurring factor appears to be SNS hyperactivity.

Results and discussion of key findings of each manuscript:

1.1. Retinal vasculature reactivity during flicker-light-provocation, cardiac stress and stroke risk in Africans: The SABPA study

The first manuscript aimed to examine novel associations between retinal vessel structure and functionality with cardiac stress markers, NT-proBNP and cTnT, in the bi-ethnic SABPA cohort – to establish a retina-heart link. It was also the first study globally to link systemic levels of cTnT with retinal vessel structure and function. Furthermore, as the urban-dwelling Africans presented a greater hypertensive prevalence profile than Caucasians (1), we hypothesized:

1. that smaller arteriolar calibres, wider venular calibres, arteriovenous-nicking (AV-nicking) and diminished arteriolar and venular dilation during FLIP, will be associated with increased cardiac stress; and
2. that an increased cardiac stress profile, AV-nicking and wider retinal venules will accompany a higher stroke risk in Africans exclusively. These findings inferred translational relevance.

The first hypothesis was accepted, as Africans presented with narrower arterioles (albeit statistically insignificant) and wider venules. Specifically, reduced retinal arteriolar calibres associated with higher cTnT levels. Attenuated arteriolar dilatory response to flicker-light-induced-provocation (FLIP), in the form of arteriolar maximum dilation and area-under the curve during FLIP (AUC_{artery}), which describes the longevity, intensity and degree of the vessel response, was inversely associated with cardiac stress (both cTnT and NT-proBNP). Finally, venular calibres were positively associated with NT-proBNP, again, in Africans exclusively.

The findings also revealed that cardiac stress markers, wider venules and AV-nicking predicted an increased, medium to high subclinical stroke risk in Africans. Hence the second hypothesis was also accepted.

Concerning the overall aim of the current thesis, *Manuscript 1* indicated that observable changes in the retinal vasculature and cardiac stress markers (cTnT and NT-proBNP) support the notion that these markers may serve as investigative tools for the prediction of cardio-and cerebral vascular risks. *Manuscript 1* thereby served to establish a more defined brain-**retina-heart** link.

1.2. Heart rate variability, the dynamic nature of the retinal microvasculature and cardiac stress: Providing insight into the brain-retina-heart link: The SABPA study

The second manuscript built on the findings of the first. This was also the first study globally to have examined the time-and frequency-domain measures of HRV during FLIP in relation to dynamic changes in the retinal vasculature and systemic levels of cTnT. In this manuscript retinal vessel structure and function, HRV time-and-frequency domain parameters during FLIP and cTnT was investigated to provide further evidence in support of the brain-retina-heart link. Again, due to the well documented SNS hyperactivity in the SABPA African group (1-7), we hypothesised:

1. that FLIP-induced changes in HRV, resembling SNS hyperactivity, increased modulation and tone and/or vagal withdrawal, will relate to attenuated retinal dynamics in Africans; and
2. that FLIP-induced changes in HRV, compatible with increased SNS activity and tone and/or vagal withdrawal, will associate with higher levels of cTnT, indicating cardiac stress, specifically in Africans.

The first hypothesis was accepted as attenuated arteriolar constriction and venular dilation to FLIP were associated with decreased DVA-FLIP HRV (time-and frequency-domain measures) in Africans exclusively. Additionally, static retinal vascular calibres were also inversely associated with DVA-FLIP HRV (time-and frequency-domain measures) in Africans. Wider venules and a smaller AVR were positively associated with increased SNS modulation (LFnu) and diminished vagal modulation (HFnu). This introduces the possibility that the FLIP-induced stress-response may persist or be prolonged due to the presence of general SNS hyperactivity in Africans. In contrast, in Caucasians, post-FLIP retinal structure (arteriolar calibres and AVR) were positively associated with both time-and frequency-domain parameters indicative of a restored SNS balance. Such maintained sympatho-vagal balance is characterized by an increase in vagal modulation (HFnu) and decreased SNS modulation (LFnu) – possibly indicating more effective neurogenic control.

Higher systemic levels of cTnT were also associated with decreased HRV during FLIP in Africans. The inverse association between systemic cTnT and rMSSD during FLIP in Africans, provides further support for the brain-retina-heart link. This link further maintains the observed association between cTnT and attenuated retinal arteriolar responses in *Manuscript 1*. The latter might link systemic levels of cTnT with vagal withdrawal and increased SNS tone in Africans. Thus the second hypothesis was also accepted.

Concerning the overall aim of the current thesis, the findings in *Manuscript 2* support that SNS hyperactivity may be associated with altered hemodynamics of the retinal and possibly the cerebral microvasculature. This may also imply that disrupted retinal (and cerebral) autoregulation may be a general result of systemic SNS dysregulation, indicating the importance of central control by the brain on microcirculatory systems in different vascular beds. Hence the SNS exerted a significant effect on smooth-muscle tone of the retinal vasculature, either directly or indirectly, solidifying a brain-retina-heart link. This Manuscript's findings may also justify FLIP as an acute stressor that elicits a SNS driven response. Additional translational value of this manuscript might theoretically imply possible increased risk for glaucotamous and optic nerve damage, due to SNS hyperactivity, in the SABPA Africans. However, no direct markers for glaucoma or optic nerve damage were assessed in this population, therefore this observation is purely based on physiological speculation.

1.3. Low Brain-derived-neurotrophic-factor reflects attenuated retinal vascular functionality and increased stroke risk: The SABPA study

As the first two manuscripts explored the myogenic and neurogenic aspects of retinal vessel dynamics, *Manuscript 3* built on these results and investigated the neurovascular coupling aspect of neurogenic control of retinal vessel dynamics. This was the first study globally, to date, that examined systemic levels of BDNF (the most abundant neurotrophic factor in the retina) in relation to retinal vasculature function during FLIP. Due to the well-documented stroke risk and attenuated

arteriolar functionality in response to FLIP, in the SABPA African group, as established in *Manuscripts 1* and *2* as well as previous SABPA studies, (1, 8), we hypothesised:

1. that lower systemic levels of BDNF will be associated with attenuated retinal arteriolar dilation and constriction during FLIP, in Africans; and
2. that an increased stroke risk will relate to lower BDNF levels, in Africans – adding translational relevance.

The first hypothesis was accepted, as attenuated retinal arteriolar variables (maximal constriction, time to constrict and arteriolar diameter after flicker cessation) were associated with lower serum BDNF levels, in Africans alone.

However, despite these attenuated responses, again being exclusively present in Africans, lower BDNF levels predicted an increased stroke risk in both ethnicities. A novel BDNF cut-point of 1.5ng/mL predicted the development of retinopathy, again irrespective of ethnicity. The second hypothesis was therefore rejected.

Regarding the overall aim of the current thesis, *Manuscript 3* indicated that low BDNF levels observed in the total SABPA cohort may indicate its neuro-protective effect might be diminished in our cohort. However, these associations and low BDNF levels may be due to the greater stress susceptibility, sustained SNS hyperactivity and modified hemodynamics reported in the African group. Specifically the modified hemodynamics might support the observed alterations in retinal vasculature smooth-muscle responses and possible endothelial dysfunction. Such a hemodynamic profile might contribute to diminished myogenic control mechanisms and disturbed autoregulation, evidenced by the attenuated arteriolar constriction responses and delayed diameter recovery, further in support of the findings reported in *Manuscripts 1* and *2*. Consequently the lower BDNF levels and associated attenuated retinal arteriolar responses reflected a possible, pre-clinical impaired neurovascular coupling, linked to increased stroke risk. These observations may be attributed to sustained SNS hyperactivity responsible for long-term, high-pressure exposure.

2. Discussion of main findings and comparison with the literature (Figure 5.1)

The SABPA study was ideally designed to assess SNS hyperactivity, in relation to cardiovascular disease risk. It is the only study in Africa perfectly suited to explore the brain-retina-heart link and the controversial idea that the SNS may directly or indirectly affect retinal vascular calibres and dynamics. The original findings of this thesis contribute significantly to the existing literature, as it is the first to assess neural control, cardiac stress and influences on retinal vascular dynamics.

SNS hyperactivity, BP increases, cardiometabolic, myocardial ischemic and stroke risk are persistently evident in the SABPA African group (1-10). In the SABPA Africans, the main contributor to the observed sustained high-pressure system, is SNS hyperactivity (1, 3, 7). This alters perfusion pressures and adequate blood flow to peripheral tissues (5). The modified hemodynamic responses observed during such high-pressure conditions, directly contribute to faulty retinal autoregulation, as the autoregulatory capabilities tend to vanish with increased BP and ocular pressures (11). A sustained high-pressure system leads to a decrease in and eventual diminishing of myogenic control mechanisms (12, 13). In *Manuscript 1* cardiac stress might be implicated in the eventual diminishing of retinal vasculature myogenic control mechanisms and disrupted autoregulation, in an attempt to expand the accommodation capacity (4, 7, 9, 14). The clinical relevance of *Manuscript 1* lies in its description of cTnT's role in stroke and cerebrovascular diseases, as the relative risk of developing stroke in the subsequent 10 years was about 50% more increased in Africans when cTnT levels were above 4.2ng/mL (OR= 1.51). The significance of this cTnT cut-point has also been consistently reported with acute and chronic stress in Africans – both attributed to sustained SNS hyperactivity. Findings in *Manuscript 1* showed that a mean hypertensive state, higher cTnT, lower NT-proBNP levels and structural changes (AV-nicking and wider venules) in retinal vasculature were all independently related to stroke risk.

Myogenic, neurogenic and humoral processes are assumed to be involved in autoregulation of retinal vascular function (15, 16). Indeed, in the SABPA African cohort, SNS hyperactivity was associated with alterations in microvascular neural nitric oxide (NO) responses (1) and increased norepinephrine levels (5, 6). Consequently the controversial link between SNS hyperactivity and altered dynamic of retinal vessels was investigated in *Manuscript 2*. In Africans attenuated retinal arteriolar, as well as venular dilatory responses, were associated with decreased HRV time-and frequency-domain parameters during FLIP. Findings implied vagal withdrawal, possibly driven by increased SNS activity during FLIP. Such attenuated responses may reflect cerebral vasculature responses, as recent investigations showed that vagal withdrawal and increased SNS activity related to poorer whole brain perfusion (17, 18). Arteriolar constriction was inversely associated with both LFnu and HFnu, indicating a decreased power in all spectral bands, typically observed during and attributed to an insufficient SNS response (19). Indeed, Malan and co-workers showed that SNS hyperactivity preceded β-adrenergic hypo-responsivity, in SABPA-Africans (5). It appears that as SNS hyperactivity increases, the ability of the retinal vasculature to auto-regulate, decreases. Generalized SNS hyperactivity may thus elicit loss of tone and/or altered hemodynamics of the retinal vasculature. Novel inverse associations between HRV parameters during FLIP and static retinal calibres also implied that if long-term SNS hyperactivity is present, impeded autoregulation may persist even after the stressor (FLIP) has ceased. *Manuscripts 2's* results support the notion that the SNS driven activity exerts a significant effect on smooth-muscle tone of the retinal

vasculature, either directly or indirectly. Furthermore, that FLIP may be defined as a stressor that elicits a SNS-driven response from the retinal vasculature, thereby impacting on its autoregulative capacity.

Findings in *Manuscript 2* support results in *Manuscript 1*, specifically within the context of cTnT's association with SNS activity and FLIP as a mental/physical stressor. The inverse association between systemic cTnT and rMSSD during FLIP in Africans provides further support for the brain-retina-heart link. Higher levels of cTnT, as well as mental stress-induced increases in cTnT were related to SNS hyperactivity in Africans (20-22). This supports our observation of an association between cTnT and attenuated retinal arteriolar responses. The latter possibly links systemic levels of cTnT with vagal withdrawal and increased SNS activity and modulation.

As the first two manuscripts explored the myogenic and neurogenic aspects of retinal vessel dynamics, *Manuscript 3* built on these results and investigated the neurovascular coupling aspect of neurogenic control of retinal vessel dynamics. This was the first study to date that examined systemic levels of BDNF (the most abundant neurotrophic factor in the retina, directly associated with SNS activity) in relation to retinal vasculature function during FLIP.

Although both our African and Caucasian groups presented with BDNF levels below the reference ranges (1.3-1.8ng/mL vs 6.97-42.6ng/mL) (23, 24), ethnic-specific associations existed between BDNF and retinal arteriolar responses during FLIP. In Africans, low BDNF levels were associated with attenuated retinal arteriolar responses during FLIP. The retinal arterioles showed greater dilation at low BDNF levels, yet with a pronounced delay in their constriction ability and delayed reduction in their diameter after cessation of FLIP. It appears that, in Africans, the lower BDNF levels might reflect disrupted neurovascular coupling. Dilation occurred, ensuring adequate blood supply, yet the counteracting constriction mechanisms displayed a delayed response (25). This may also indicate a prolonged retrograde propagation of the vascular response, possibly influenced by sustained SNS hyperactivity. Again, greater stress susceptibility and sympathetic hyperactivity (1-8) reported in this group may result in lower BDNF levels and modified hemodynamics. BDNF's modulating abilities will directly be altered by a sympathetic hyperactive state, possibly reflected by the decreased levels in BDNF (23, 26, 27). Indeed, BDNF has been linked to activity in the cingulate and medial prefrontal cortex, possibly contributing to modulating the effects of SNS hyperactivity (26). Yet when sustained SNS hyperactivity persists, a diminished and eventual decrease in BDNF may ensue. Specifically the latter might support altered retinal vasculature smooth-muscle responses and endothelial dysfunction (6, 23). Such a hemodynamic profile might contribute to diminished myogenic control mechanisms and disturbed autoregulation, evidenced by the attenuated arteriolar constriction responses and delayed diameter recovery, supported by the findings in *Manuscripts 1* and *2*.

Interestingly, despite the low BDNF levels, in the Caucasian group, it was not associated with attenuated arteriolar constriction time or the average arteriolar diameter after flicker cessation. Besides BDNF's neuroprotective effect, it also plays a central role in energy homeostasis, and has a pronounced anti-diabetic effect (28). However, it is possible that in Caucasians, low BDNF levels may also adversely affect glucose metabolism and tissue sensitivity for glucose. Regardless of the ethnic-contrasting associations observed, lower BDNF levels predicted an increased 10-year stroke risk with an odds ratio of 1.56, in the total cohort. Low BDNF levels were linked to a poorer long-term functional outcome following ischemic stroke (29).

The neuro-protective effect of BDNF might be diminished to a certain extent in both SABPA Africans and Caucasians, accompanying an increased 10-year stroke risk, albeit via different mechanistic actions. BDNF's direct action on vascular smooth-muscle cells might be detrimentally modified, altering arteriolar vascular resistance and contributing to disturbed neurovascular coupling and increased stroke risk.

Overall, the African risk profile may mainly be driven by SNS hyperactivity, higher circulatory pressure and cardiac stress resulting in the loss of myogenic control, neurogenic tone, neuroprotective influences as well as disrupted neurovascular coupling. As a consequence the autoregulatory capacity of the retinal microvasculature will be impaired and the risk for stroke, retinopathy and possibly optic nerve and glaucomatous damage increases within this population. The risk profile in Caucasians, albeit speculative, might be driven by factors essentially impacting on and contributing to glucose metabolism and acute stress, exciting factors that remain to be explored within the Caucasian group.

3. Chance and confounding factors

Chance and confounders

The previously discussed results and conclusions are assumed credible, however one should take into account the possibility of chance factors that might influence the validity of these results and subsequent conclusions. Despite rigorous efforts and control measures, stepwise regression analyses indicate that five percent of all statistically relevant correlations might be due to chance. It is important to critically reflect on specific factors that may have influenced or confounded the results.

In all manuscripts we applied a hypothesis-driven approach where higher sympathetic activation and stroke risk were shown in the SABPA Africans. Interaction testing revealed significant differences between Africans and Caucasians but not between gender groups independent of selected *a priori* covariates. Therefore ethnicity was defined as the grouping variable in all analyses.

Statistical analyses of all three manuscripts were adjusted for carefully selected *a priori* covariates. For each manuscript, individual analyses were performed to determine which variables significantly impacted on the specific sub-population chosen for that particular manuscript. *A priori* confounders for each were determined as:

Manuscript 1: Retinal vasculature reactivity during flicker-light-provocation, cardiac stress and stroke risk in Africans: The SABPA study - age, body surface area (BSA), cotinine, gamma-glutamyl transferase (γGT), HbA_{1c}, total cholesterol/HDL cholesterol ratio, tumour necrosis factor alpha (TNF-alpha), hypertensive/diabetic retinopathy, 24h pulse pressure; Additional adjustments were made for CRVE in the CRAE model and vice versa throughout *manuscript 1* and *2*. For NT-proBNP models, thyroxin and triiodothyronine were added as covariates. Additional sensitivity analyses were conducted to determine the influence of angiotensin-converting-enzyme inhibitors effect in NT-proBNP models. Aside from SABPA-exclusion criteria, additional exclusion criteria for all analyses pertaining to *Manuscript 1* included: epilepsy, unusable retinal vessel recordings and incomplete dynamic vessel data.

Manuscript 2: Heart rate variability, the dynamic nature of the retinal microvasculature and cardiac stress: Providing insight into the brain-retina-heart link: The SABPA study - age, BSA, cotinine, γGT, HbA_{1c}, total cholesterol/HDL cholesterol ratio, nocturnal dipping status (as it influences SNS tone and modulation) and 24h pulse pressure. Aside from SABPA-exclusion criteria, additional exclusion criteria for all analyses pertaining to *Manuscript 2* included: epilepsy, arrhythmia, unusable retinal vessel recordings, incomplete dynamic vessel data and HRV data during FLIP.

Manuscript 3: Low Brain-derived-neurotrophic-factor reflects attenuated retinal vascular functionality and increased stroke risk: The SABPA study - age, gender, γGT, HbA_{1c}, TNF-alpha, total cholesterol/HDL cholesterol ratio, diastolic ocular perfusion pressure and hypertensive/diabetic retinopathy. Additional exclusion criteria for *Manuscript 3* included: epilepsy, arrhythmia and unusable retinal vessel recordings, incomplete dynamic vessel and BDNF data.

Vessel segment diameter was *not* added as a co-variate to any dynamic analyses, for the baseline-value is already taken into account by the percentage change formula applied to obtain dynamic parameters.

Additional care was taken to ensure that no variables with a known physiological interdependence were ever added simultaneously into the same model, thereby successfully avoiding co-linearity.

Furthermore, for each manuscript sensitivity analyses were performed. Forward stepwise regression analyses, with the same set of covariates were repeated in several models in both ethnic groups.

Excluding participants with diabetes, on diabetic treatment and those using any form of hypertensive treatment, did not influence the outcome in any of the three manuscripts.

The aforementioned adjustments and exclusions were done to diminish erroneous interpretation of results. This added to the overall value of the final results obtained. The latter, together with the well-controlled setting and design of the SABPA study, with its standardised protocols, specifically designed to measure SNS activity, and small intra-inter variability recorded within these procedures.

Strengths

- The SABPA study is a target population study which has been designed to present individuals of both investigated ethnicities (Africans and Caucasians) equally and ensured equal distribution of gender and socio-economic status.
- The average age of the investigated study population was (± 49 years). This allowed us to investigate FLIP induced retinal vascular changes between ethnicities from a similar working environment. It is important to note that reaction time might modify with age, but the manner, or reaction profile/pattern remains the same.
- All measurements were conducted under extremely well controlled scientific conditions, the Metabolic Research Unit, ensuring utmost environmental stability, such as temperature control, humidity and environmental exposure.
- All measurements were conducted between February and May 2011 and 2012, so as to avoid any seasonal variations.
- A precise, standardized protocol was followed with regard to all measurements, analyses and sample management.
- DVA protocol was ideally designed to allow for the investigation of sustained SNS activity on retinal structural parameters (Dynamic analyses were performed prior to static).
- Blood samples were immediately stored at -80ºC to ensure sample stability and integrity.
- Individual, additional exclusion criteria for each manuscript e.g. epilepsy, arrhythmia, unusable retinal vessel recordings etc. ensured a study sample free of apparent bias and not subject to statistical anomalies.
- Great care was taken to identify confounding variables, based on statistical and physiological relevance and inference.
- All retinal vessel analytical data were captured and interpreted by 2 experienced scientists and all medical, clinical and pathological classifications were done by a board certified ophthalmologist.
- With particular concern to *manuscript 2*, the DVA protocol was ideally designed to investigate the residual effect of SNS evoked activity on static retinal parameters.

Limitations

- One of the most important limitations of the current study is the cross-sectional design. Therefore we could not directly infer causality and conclusions were made after careful consideration of known literature and physiological viability.
- The total study sample for this study was quite small, after considering the aforementioned exclusion criteria; *Manuscript 1* (N=317); *Manuscript 2* (N=264); *Manuscript 3* (N=280). However, the necessary statistical power was still reached.
- The conclusions made in this study *only* pertain to two specific ethnic and socio-economic groups from a very particular demographic area in South Africa – a country rich in ethnic and cultural diversity. Hence these results cannot be extrapolated and applied to the entire South African population (30).
- Regarding clinical inference, the risk profile differed distinctly between our African and Caucasian teachers, consequently we were unable to evaluate whether the differences are ethnic specific, or whether the results are due to the observed higher risk in the African cohort. Unfortunately, the sample size of pair-matched risk profiles was too small to perform meaningful statistical analyses.
- The greater retinal arteriolar dilation reported in Africans compared to Caucasians might possibly be due to an inherent *greater dilatory capacity*, as the arterioles are reported to be narrower (thus not necessarily an advantageous dilation) (1). This might imply that the dynamics observed may reflect retinal arteriolar dysregulation and not necessarily dysfunction (please refer to the Glossary (*Appendix B*)).
- Describing the retinal profile of the SABPA-cohort was not the main aim of this study and this profile is to be explored by Smith et al (2019).
- The biological clearance rate of compounds (e.g. BDNF) might account for the lower levels observed in the entire cohort. This may be investigated in future studies.

Recommendations for future research

The research conducted to compile this study revealed several aspects that may be explored or taken into account when performing future studies. These recommendations will be categorised by technique, sampling or method as follows:

Retinal vessel analyses

- Performing saliva sampling before and after FLIP and analysing these samples for specific biomarkers (described in sections to follow), therefore acute stress sampling. (Due to the

invasive nature of blood sampling an acute stressor or FLIP, blood sample will not be practically feasible, for the patient/ participant will experience severe discomfort).

- Performing additional retinal scans to survey the individual layers of the retina (e.g. spectral-domain optical coherence tomography, confocal laser ophthalmoscopy, scanning laser polarimetry and retinal thickness analysis), thereby assessing the condition of the neural retinal layers, ganglion cell layers and condition of the blood-retina barriers.
- Assessing baroreceptor sensitivity during DVA and FLIP analyses.
- Determining the pulse wave velocity and amplifications of the retinal microvasculature and changes therein during FLIP.
- Performing simultaneous DVA and electroencephalogram (EEG) analyses, to determine cerebral cortical activity elicited by FLIP, to further elucidate the type of cortical response elicited.
- Future retinal vessel investigations (as well as FLIP) to include beat-to-beat BP monitoring via e.g. the Finometer.
- Additionally exploring the particular grade of retinopathy (Grade 1-4), based on the defined characteristics as stated by Schrieffer, and possible stroke risk.
- Deriving an applicable algorithm capable of correlating specific retinal vascular dynamics with static calibres, should be explored as a possible translational, diagnostic tool.

Biomarker assessment

- Analysing resting blood samples for markers of gliosis in the retina to afford more clarity on the role of the glial cells in the retina as well as stroke risk (e.g. S100b (apoptotic injury associated with depression, anxiety and acute brain injury), glial cell-derived neurotrophic factor (indicative of blood-brain/ blood-retina barrier health and plasticity), neural growth factor (associated with blood-brain/ blood-retina barrier condition and glial cell health), glial fibrillary acidic protein (GFAP) (plays a key role in neuron-astrocyte interactions, may reflect the state of neurovascular coupling and glial scarring), laminin (essential structural component in the basal membrane, influences cell migration, differentiation and adhesion, might impact on retinal vessel dynamics) and neuregulin (essential in glial cell health, speculated to play a modulating role in anxiety and depression, also related to cardiomyocyte injury, growth as well as central and peripheral calcium balance).
- Investigating the clearance rate of the aforementioned biomarkers, to determine whether acute and chronic changes are attributed to short/long-term-mediated processes.

Heart-rate-variability assessment

- Exploring the use of additional HR parameters, such as the HRV index, which depends on wavelength entropy measures. Once the wavelet coefficients are obtained, the energy for each coefficient is calculated as described in the literature. After calculating the normalized values of wavelet energies, which represent the relative wavelet energy (or the probability distribution), the wavelet entropies are obtained using the definition of entropy.
- Improving algorithms that allow for the assessment of transient changes in the RR interval, to more effectively relate the physiological nature of these HRV variables.
- Trigonometric analyses of the RR intervals for smaller selected windows during FLIP (>5min) should be performed (31).

Study design

- Repeating the current study with a longitudinal approach may establish cause and effect mechanisms.
- All procedures and assessments can be repeated in a larger population-based study that includes different ethnic and cultural groups from various demographics, expressly to establish the validity of our conclusions and findings, specifically in Africans.
- Several investigations have identified increased psychosocial stress experienced by this vulnerable African cohort, as one of the main contributors to this observed higher risk profile. However, the exploration of psycho-social stress influences was not within the scope of the current investigation and we suggest assessing the effect of psychosocial stress on the structure and function of the retinal vasculature, cardiac stress markers and stroke risk.
- Medication usage will most certainly affect neural control, cardiac stress and retinal dynamics. Specifically hypertension medication, like calcium channel blockers have been reported to cause reflexive increases in sympathetic activity, increasing stroke and myocardial ischemic risk. Angiotensin-conversion-enzyme (ACE) inhibitors also influence the volume-loading conditions in the cardiovascular system. Each medication type should therefore be assessed individually to determine their effect on the variables investigated in this study.

4. Conclusion

The current study exemplifies the possible role of SNS hyperactivity, regarding the control of retinal vascular dynamics, specifically in Africans. This role is emphasized as SNS hyperactivity and the subsequent modified hemodynamic responses observed during such high-pressure conditions, chronic cardiac stress and diminished neuroprotective effects may directly contribute

to impaired retinal autoregulation. This sustained high-pressure system may not only lead to a decrease in and eventual diminishing of myogenic control mechanisms, but may also indicate an altered neurogenic response and neurovascular coupling, all ultimately contributing to autoregulation. The presence of pre-existing SNS hyperactivity, and additional SNS activity provoked during FLIP further increases SNS activity and modulation. During a stressor, like FLIP, an existing hyperactive SNS (as is repeatedly evident in the SABPA Africans) will further be challenged, thereby causing further reduction in HRV indicating greater SNS activity and modulation, possibly in an attempt to maintain adequate perfusion. Existing SNS hyperactivity-driven, pressure-induced endothelial dysfunction and impaired smooth-muscle responses may manifest as attenuated retinal vessel dilatory and constriction responses. The latter also indicates that BDNF's direct action on vascular smooth-muscle cells might be altered, attenuating arteriolar vascular resistance and contributing to disturbed neurovascular coupling. This increased, sustained SNS activity/modulation may lead to a prolonged vessel response, even after the stressor has ceased, indicating difficulty in re-establishing proper SNS balance, once this balance has been chronically disturbed. As a consequence the autoregulatory capacity of the retinal microvasculature will be impaired and the risk for stroke, retinopathy and possibly glaucoma and optic nerve damage risk is increased within the SABPA African population (**Figure 5.1**).

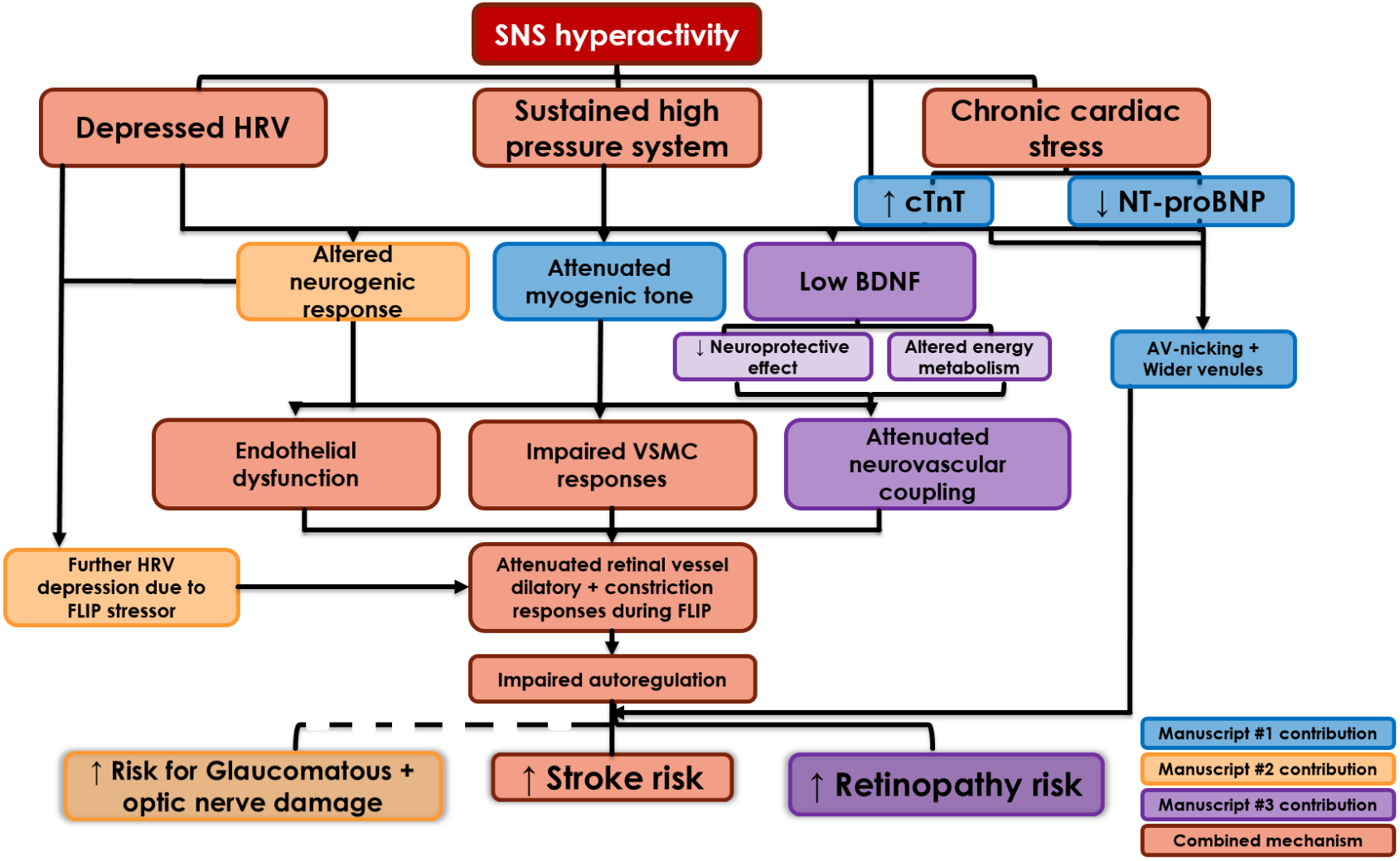

Figure 5.1: Proposed mechanistic action of a hyperactive SNS's effect on retinal vessel structure and function. Where: SNS, sympathetic nervous system; cTnT, cardiac troponin T; NT-proBNP, Amino-terminal pro-B-type natriuretic peptide; BDNF, brain-derived neurotrophic factor; HRV, heart rate variability; FLIP, flicker-light-induced-provocation; VSMC, vascular smooth muscle cells; ↑ increase; ↓ decrease.

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Appendicies

Supplementary Materials and Methods

Study Design

This sub-study was mainly nested in the second phase of the prospective Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study, which was conducted between late summer and late autumn of 2011 and then again in 2012, as to avoid seasonal variations (1).

Research Participants

For the first wave, all teachers enrolled in 43 schools (N=2170) of the Dr Kenneth Kaunda Education District, North West Province, South Africa, were invited to participate (**Figure S1**). Power analyses were performed for the SABPA study cohort by using previous studies for autonomic dysfunction to obtain relevant effect sizes based on differences in biological profiles. Resulting sample sizes of 50 – 416 enabled explanation of biological differences with a statistical power of 0.8, and level of significance of 0.05. The target population included urban-dwelling well-educated Black (African) and White African (Caucasian) male and female teachers. This exclusive selection of teachers ensured a socio-economic-education equated sample from a similar working environment; cultural differences could not be excluded. All volunteering teachers had medical aid benefits and were screened to meet study eligibility criteria during the recruitment phase (**Figure S1**). Those complying, formed the respondent group of 409, but those not complying, formed the non-respondent group (N=62). Data are currently available for 409 teachers of phase 1 and all were invited to partake in phase 2 from which 359 were followed up in phase 2. The participants voluntarily took part in the study and were initially recruited as part of phase 1 of the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study. All participants included in phase 1 (2008-2009) were invited to take part in phase two (2011-2012). Of the initial 409 participants, 359 reported for the second phase of the study. Only participants, who took part in the second phase of the study, were included for this study, as no retinal measurements were taken during the first phase. All teachers from different schools were informed about the aims of the study and written informed consent was obtained before their inclusion in the study. As they were volunteers, any participant was free to withdraw at any stage of the investigation. Specific exclusion criteria for phase 1 of the SABPA study were however: alpha/beta blocker users, any psychotropic drug (eg. anti-depressants), pregnant or lactating women, blood donation or vaccination 3 months prior to the investigation and tympanum temperatures of greater that 37.5^{o}C.

Phase 2's study sample comprised of urban African and Caucasian, male and female teachers (n=359) from the North-West Province, South Africa, aged between 20 and 65 years (**Figure S1**). The motivation behind the selection of teachers only was to obtain a socio-economic-similar sample from a similar working environment; cultural differences could however not be excluded. Additional exclusion criteria for this sub-study included atrial fibrillation, ventricular ectopic episodes, epilepsy and poor quality retinal vessel recordings. All ectopic episodes were excluded from the dataset through manual exclusion as identified by calculated QQ-and scatterplots. Each manuscript had further exclusion criteria influenced by the main variables and research questions.

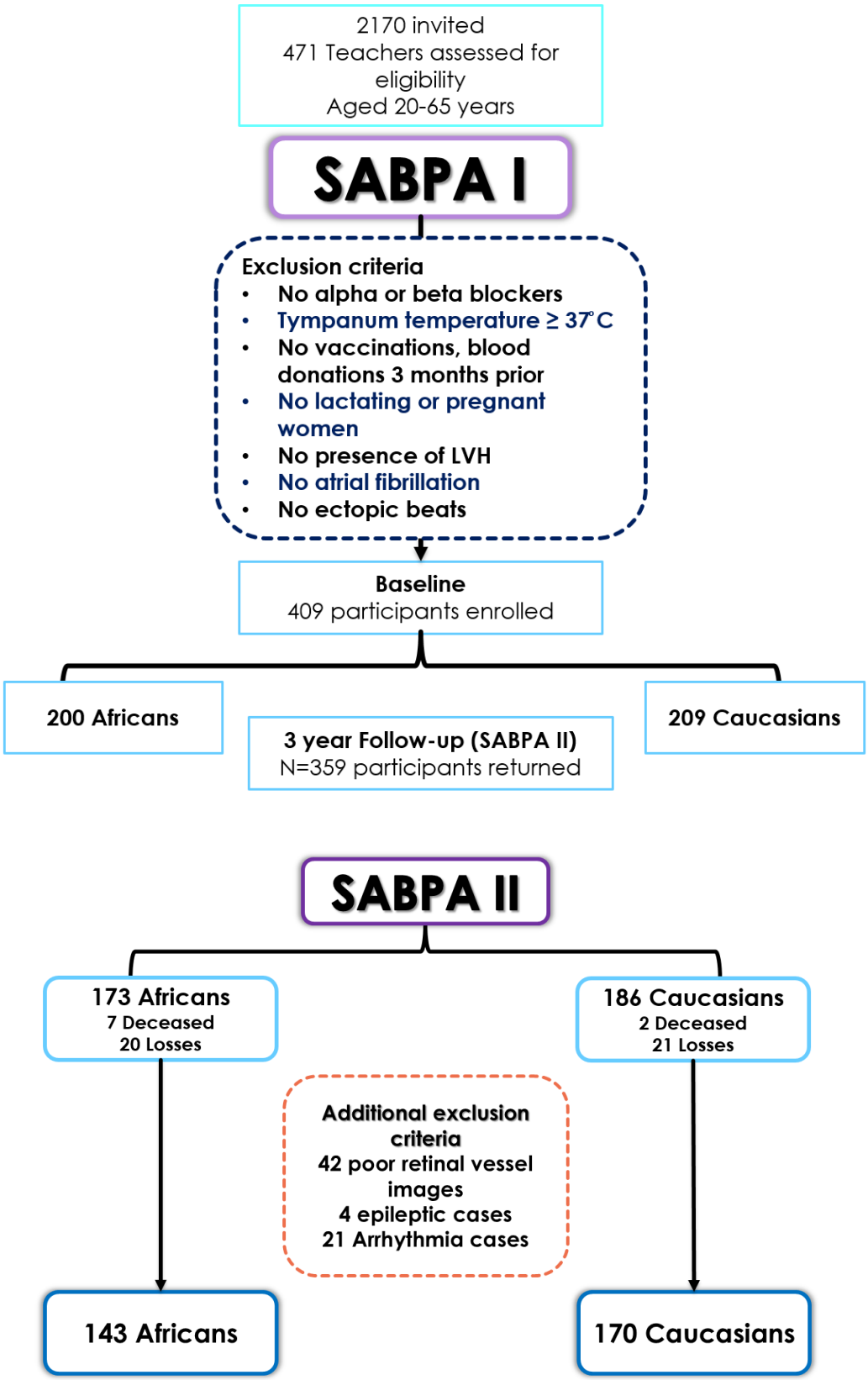


Figure S1: The design of the SABPA bi-ethnic prospective cohort

Ethical Considerations

Ethical approval, for this sub-study, was obtained from the Health Research Ethics Committee (HREC) of the North-West University, Potchefstroom Campus (Ethics number: **NWU-00057-17-A1, please refer to the ethics certificate Appendix D**). The SABPA study had obtained ethical approval as well as extended approval for the second phase (SABPA ethics number: **NWU-00036-07-S6, please refer to the ethics certificate Appendix C**). Written informed consent had been obtained from all volunteers prior to participation. All procedures adhered to the applicable institutional guidelines and terms, as stated by the Declaration of Helsinki of 1975 (version revised in 2004), both in 2008-2009 and 2011-2012.

Trained Black African fieldworkers, including a clinical psychologist and trained post-graduate students, were involved during the completion phases of SABPA. They were mainly tasked with recruiting participants, ambulatory blood pressure measurement (ABPM) and ECG measurements, urine sampling, provision of the standardized dinner, completion of psychosocial battery, general health and medical history questionnaires. Individual feedback was specifically conducted by the Principal investigator and clinicians, as per ethical standards and regulations.

Recruitment of Participants

The headmasters of schools were informed about the SABPA project as their cooperation and support were vital in the execution of the project. The principal investigator of the SABPA study, together with a representative of that specific community was tasked with recruiting and informing participants. The community representative was tasked with informing and conversing with the headmasters about the details of the project.

Informed Consent

Individuals who refused participation in the study, or those who withdrew from the study at a later stage, were thanked for their involvement and were not troubled again. The samples taken from the individual voluntary participants were screened only once by registered nurses to assess compliance with the inclusion criteria. Recruitment, screening and informed sessions with the participants were performed two months prior to the study and informed consent forms were voluntarily signed after these sessions were completed. The specific details of the project were discussed with all voluntary participants, who complied with the inclusion criteria for the study. These details were discussed in English or in their language of preference. This discussion included what the objectives of this study were and what its completion hoped to achieve, what procedures would be followed and what would have been expected from each of them (e.g. resting blood pressure procedures and fasting urine and blood samples are required, importance of correct sampling methods, incentives, staying overnight).

The participants were granted the opportunity of inquire if any of the aforementioned processes were not explained or understood well.

Signed consent forms and hard copies of original data (including any information that may identify the participants) are stored securely inside a locked cabinet in a locked room in the Hypertension Research and Training Clinic. Only the Principal Investigator has access to this information. The cohort data and samples are embedded in a single research Centre at the research institute of the Hypertension in Africa Research Team, North-West University (Potchefstroom Campus), South Africa. Datasets were and remain password-protected and electronic participant records stored for a maximum of 15 years in the secured facility.

Dissemination of Results

All individual results are available at all times to any inquiring participant – limited, of course to their own data, not that of any other participant. Such information may and can only be given by the Principal Investigator of the SABPA study, as they have sole access to records pertaining to personal information.

Where the confidentiality of information is concerned, Ms A. Wentzel has signed a confidentiality agreement, and the dataset that was received did not contain any variables that could identify any participant.

Risks and Benefits

Due to the nature of this sub-study, which mainly entailed the assessment of previously collected data, additional biochemical analyses of previously collected biological samples, there were neither physical nor confidentiality-breeching risks to the participants of the SABPA study. The purpose of this study is to identify and determine specific associations between the selected variables in an attempt to simplify CVD and stroke risk stratification in a South African population. Therefore participants might benefit long term, and contribute to the community. However they did not directly gain any benefit from this sub-study.

General procedure of investigation

Clinical assessments were done over a two-day period during the second phase or follow-up – similar to baseline. Before 08h00 of the first clinical assessment day, two participants were each fitted with an ambulatory blood pressure (ABPM) and 2-lead ECG monitor device (Cardiotens CE120®; Meditech CE120; Meditech, Budapest, Hungary), as well as an Actical® accelerometer to attain physical activity measurements. This device also obtained event BP prior and after retinal vessel assessments. A 24-hour standardized diet commenced and participants subsequently continued with

their normal daily activities, reporting any peculiarities such as nausea, headaches, visual disturbances, palpitations, fainting, stress and physical activity, on the issued 24-hour diary cards. At 15h00, participants were transported to the NWU Metabolic Unit Research Facility for clinical measurements including the retinal vessel imaging. Upon arrival, participants were familiarized with the experimental setup.

Participants were advised to go to bed at 22h00, fasting overnight. After the last programmed blood pressure reading had been recorded at 06h00 the following day. The Cardiotens CE120® and Actical® apparatus were disconnected, followed by anthropometric measurements. After a resting period of 30 minutes participants remained in a semi-recumbent position, then fasting blood sampling commenced. Participants also received confidential feedback on immediate available clinical measures and were referred to a physician by a registered nurse, if deemed necessary. Once all clinical measurements were completed, participants were served breakfast after which they were transported to their designated teaching facilities.

Cardiovascular Measurements

Ambulatory blood pressure was measured with the Cardiotens CE120® (Meditech, Budapest, Hungary), validated by the British Hypertension Society (BHS), which was fitted with a suitable cuff size, to the non-dominant arm of each participant. The device was programmed to ideally measure blood pressure and ECG variability in 30 minute intervals during the day (08h00-22h00), and 60 minute intervals during the night (22h00-06h00). The data was subsequently analysed by means of the CardioVisions 1.19 Personal Edition Software (Meditech®). As stipulated by the European Society of Hypertension (ESH) guidelines, a successful inflation rate is ≥ 70% (2). Our rates were 85.99% (± 9.29) and 91.80% (±7.95) in Africans and Caucasians respectively. According to the ESC guidelines; hypertension was classified as an ambulatory SBP ≥ 130mmHg and/or DBP ≥ 80mmHg (2). Additionally, day-and-night time hypertension was defined, according to the current ESH guidelines, as SBP ≥ 135mmHg and/or DBP ≥ 85mmHg (daytime HT) and SBP ≥ 120mmHg and/or DBP ≥ 70mmHg (night time HT), respectively (2) and 24-hour pulse pressure (PP), reflective of a hyperpulsatile pressure, was calculated as 24-hour SBP – 24-hour DBP, as a measure of arterial stiffness (2). The Finapres (Finapres Measurement systems©, Amsterdam, The Netherlands), validated for relative changes in BP, was utilized to calculate the stroke volume (SV) and Windkessel compliance (Cwk) via the Beat-Scope version 1.1a software package. The Cardiotens CE120® (Meditech, Budapest, Hungary), validated by the British Hypertension Society, obtained 24H-BP and continuous 2-channel ECG recordings (3).

Retinal Vessel Analyses (functional and structural)

Participants abstained from caffeinated and alcoholic beverages, exercise and food consumption at least 1 hour prior to the measurements. They were familiarized with the experimental setup and were examined for acute angle anterior chamber glaucoma risk with a pen-light by a registered nurse. All Retinal vessel analyses were done with the IMEDOS Retinal vessel analyzer (RVA) (Germany), fitted with a Zeiss FF450^{plus}) camera and the 1) VesselMap 1 and Visualis software (static images); 2) Version 3.10 software (dynamic analyses). Dynamic vessel analysis with flicker stimulation was performed first, during which pre- and post-FLIP BP measurements were manually taken, followed by image capturing for static vessel analysis (**Please refer to Figure S2**). In static vessel analysis we calculated the central retinal artery and vein equivalent (CRAE and CRVE respectively) and subsequently determined the arterio-venous ratio (AVR) as described in the section on *Static retinal analysis*.

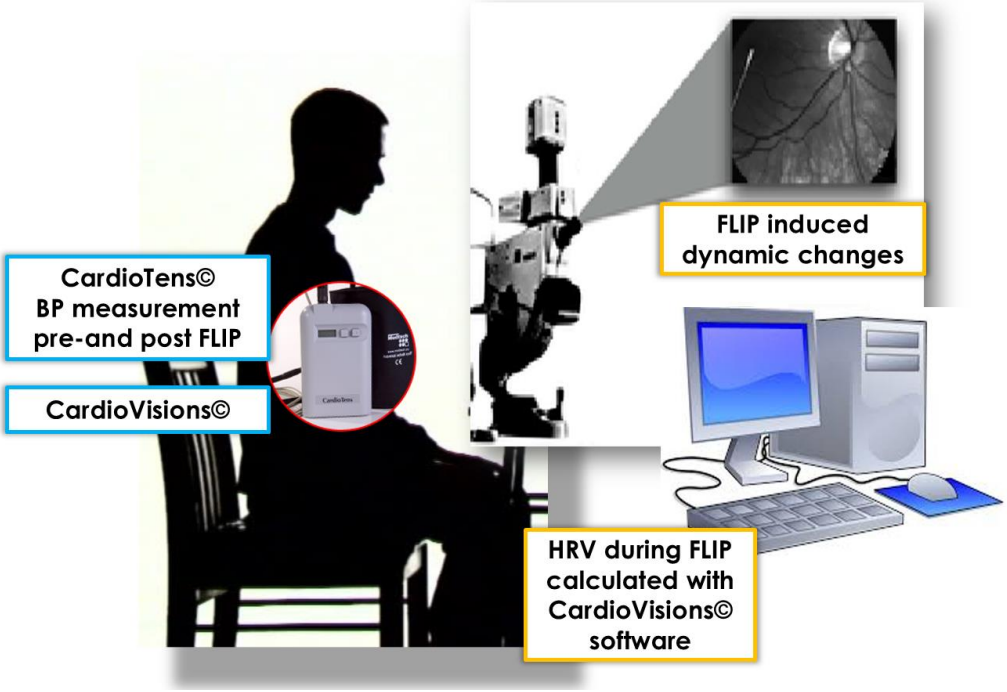
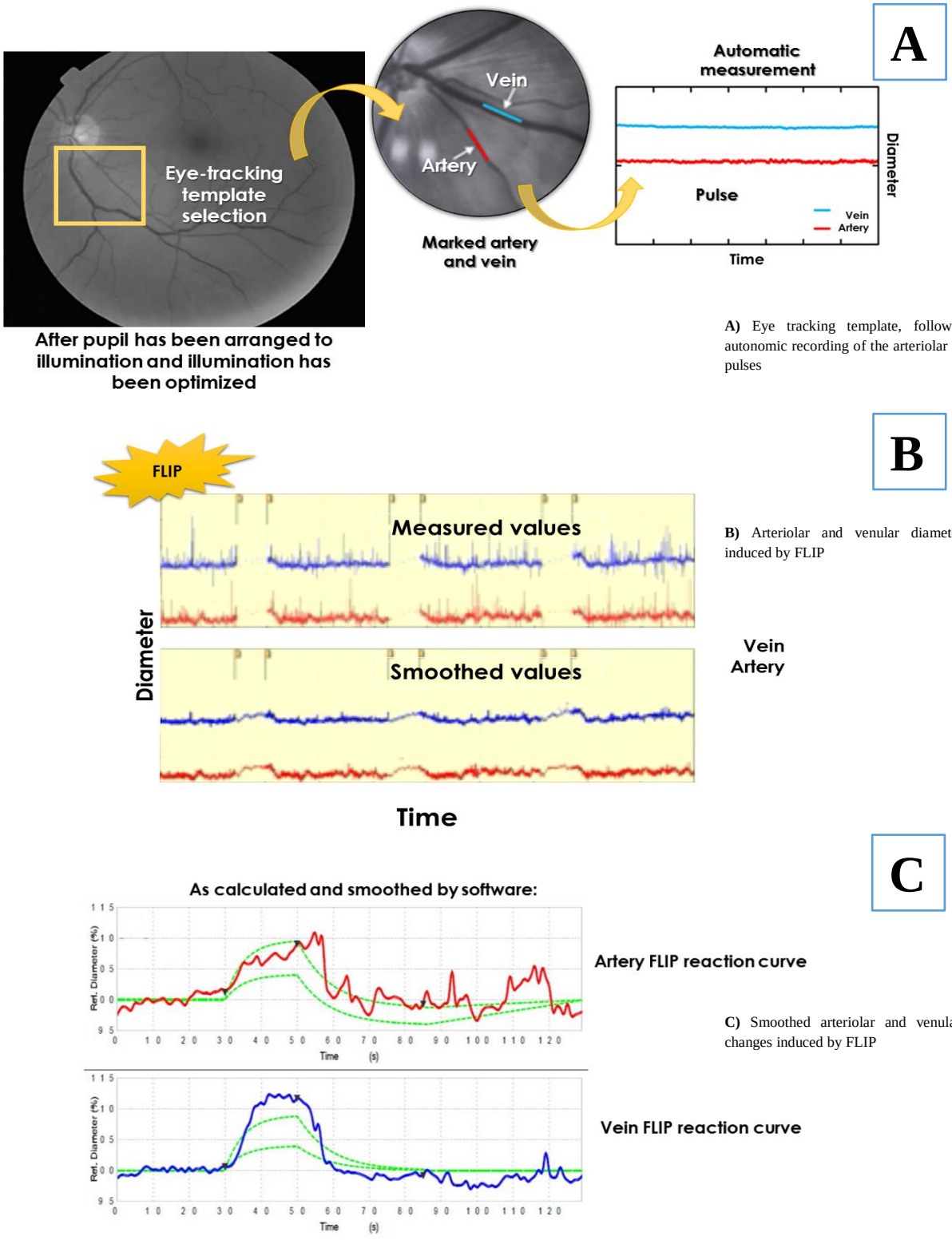


Figure S2: Retinal vessel analyses setup during Phase 2 of SABPA

Dynamic retinal analyses were completed using a standard flicker protocol by IMEDOS Systems as described by (4-6). Fifteen minutes prior to the measurement, a drop of Tropicamide (1% Alcon 1% tropicamide and 0.01% benzalkonium chloride (m/v)), was used to induce mydriasis in the right eye. The majority of participants' right eye was used. However, in cases where the right eye was unsuitable, the left eye was used (n=4). The pupil was fully dilated before retinal vessel analyses were performed. Illumination was adjusted accordingly, followed by setting the eye-tracking template (**Figure S3**). The camera was set at a 30°degree angle with the participant focusing on the

tip of a fixation rod, and an artery and vein segment (as long as possible) were primarily selected in the upper or lower temporal quadrant of the fundus image. This selection was about 0.5-2.0 optic disc diameters away from the margin of the optic disc (**Figure S3)**, followed by the FLIP period.



A) Eye tracking template, followed by the autonomic recording of the arteriolar and venular pulses

B) Arteriolar and venular diameter changes induced by FLIP

C) Smoothed arteriolar and venular diameter changes induced by FLIP

Figure S3: The DVA capturing procedure

During FLIP, the duration of the baseline was 50 seconds, followed by a 20-second flicker period and an 80-second recovery (also referred to as second baseline) period. There were 3 flicker cycles in total, lasting an added total of 350 seconds for the entire measurement. The quality of the FLIP measurements for each participant was assessed subjectively using a newly developed, previously described (5, *Smith et al, 2019 submitted*), scoring method. FLIP measurements were included provided that: 1) Analysis of the raw data was at least possible, 2) at least one good flicker was present (baseline/FLIP pattern), 3) measuring points during the flicker were consistent, 4) there was low noise and 5) there were almost no gaps in the measurement curve. A score was given (0, 0.5 or 1) for each category and each of 5 categories contributed evenly for a maximum of 5 points (0 = poor quality and 5 = excellent quality; in extreme cases a negative score could be allocated). In the current study, we only included participants with a vessel measurement score equal to or higher than 2.5.

Although the participants were instructed to remain still and to focus on the fixation rod, practical reasons for unusable retinal measurements were mainly the excessive movement of the participant during flicker light. Some participants also complained about the brightness of the light. This hindered the uninterrupted analyses of dynamic changes in the selected artery and vein segment, causing gaps in the measurement curve and inconsistencies in the measuring points during flicker. Thus such measurements did not adhere to the quality control scoring method described above. In some cases, participants found the flicker light very uncomfortable and requested for the measurement to cease. Hence, no reliable dynamic retinal data during flicker could be documented.

Absolute vessel diameters (measured in standardized measuring units (MU)) of the vessel segment was determined for each measurement. Each were calculated individually as the median value over the last 30 seconds of the first baseline phase prior to FLIP. Parameters derived from the smoothed averaged curve during FLIP, used in the current study, included: 1) the percentage maximal dilation in response to FLIP ($MD_{arteriole}$); 2) The area under the reaction curve during FLIP (AUC) was determined for arteries and veins and calculated as the percentage change per second (%.s). The latter provided information on the curve form during FLIP (0-20s), and theoretically describes the longevity, time and intensity of the vessel's response. For the values under the 100%-line the area was negative; 3) The percentage absolute maximal constriction after FLIP (MC) was the minimum value occurring after maximum FLIP-induced dilation and expressed a percentage from baseline (**Figure S4**).

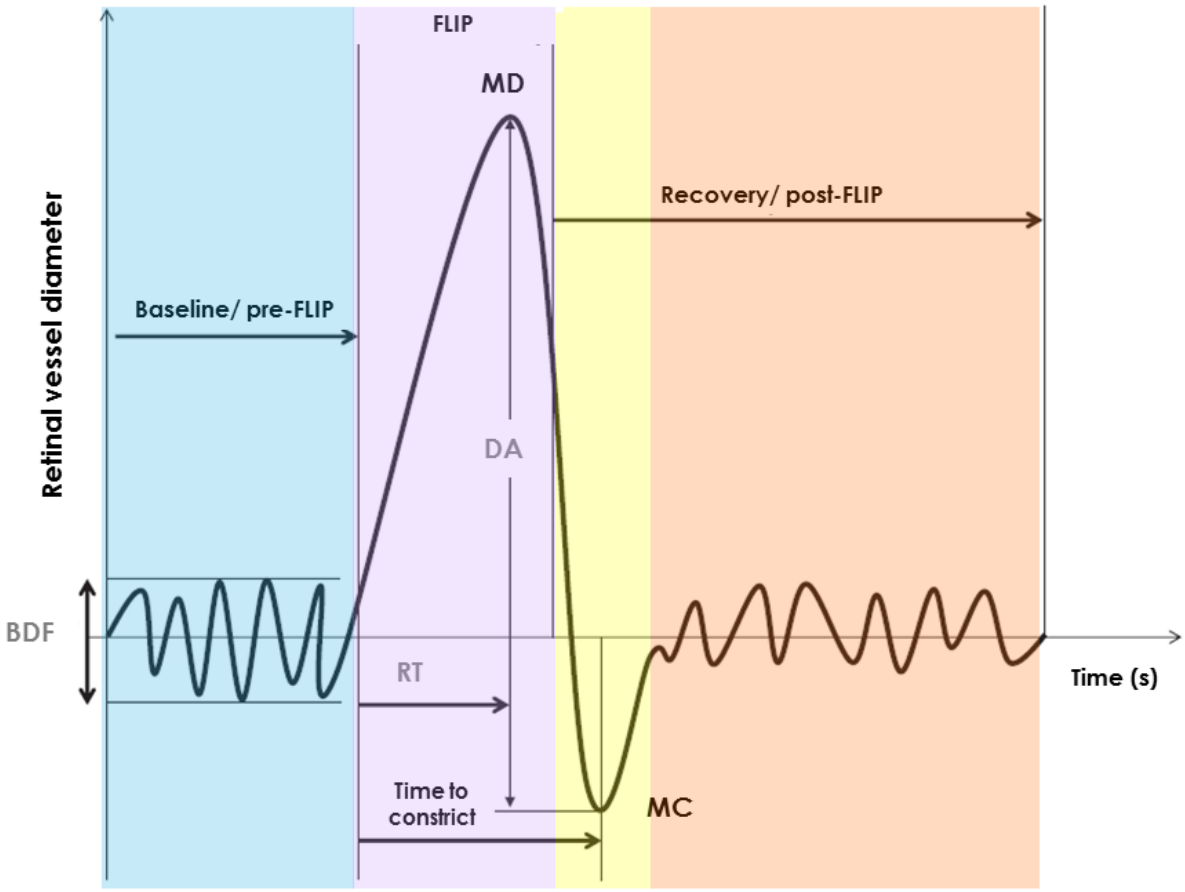


Figure S4: Schematic representation of a single cycle of dynamic vessel recording, before, during and after FLIP. Where BSD, baseline diameter fluctuation; MD, maximum dilation; RT, reaction time; MC, maximum constriction

*Dynamic Vessel Analyses calculations (**Figure S3c and Figure S4**)*

The raw numerical data generated from the retinal vessel analyzer (RVA) was exported to an Excel template (Microsoft) with macros to allow further filtering, processing and analysis of the data. As described by Smith et al (2019 *submitted*) data were extracted from all three FLIP curves and median values were calculated for each 1-second time point, and average summary curve of one cycle. The average curves were smoothed by calculating a running median with a window covering 5 seconds.

Absolute vessel diameters (measured in standardized measuring units (MU)) (7) of the vessel segment were selected for each measurement. Each were calculated individually as the median value over the last 30 seconds of the first baseline phase prior to FLIP. Parameters derived from the smoothed averaged curve during FLIP, used in the current thesis and affiliated manuscripts, are presented in the following table as adapted from Kotliar et al (8):

Table S1: Arteriolar and venular parameters derived from the smoothed averaged curve during FLIP

Parameter	Abbreviation	Calculation
Arteriolar parameters		
Mean percentage maximum dilation in response to FLIP of the arteriole	MD (% from baseline)	Calculated as the absolute maximum of the curve
Area under the reaction curve during FLIP (AUC) for arteriole	FLIP AUC arteriole (%.s)	Providing information on the curve form during FLIP (0-20s).
Time to maximum arteriolar dilation	Time to MD (s)	Assumes FLIP initiation at 0s
Mean percentage maximum constriction in response to FLIP of the arteriole	MC (% from baseline)	Calculated as the absolute minimum of the curve. For the values under the 100%-line the area was negative.
Time to maximum arteriolar constriction	Time to MC (s)	Assumes FLIP initiation at 0s
Arteriolar post-FLIP value	Arteriolar post-FLIP value (%)	The arteriolar diameter after 50-80s after flicker-light-cessation
Venular parameters		
Mean percentage maximum dilation in response to FLIP of the venule	MD (% from baseline)	Calculated as the absolute maximum of the curve
Area under the reaction curve during FLIP (AUC) for venule	FLIP AUC venule (%.s)	Providing information on the curve form during FLIP (0-20s).

Static Retinal Vessel Analyses

Static retinal vascular calibres were measured from monochrome images. First-order vessel branches were selected within 0.5 and 1 optic disc diameters from the margin of the optic disc (**Figure S5**). The VesselMap 2, version 3.02 software automatically delineated the vessels' measuring area upon selection (7). A colour retinal image was used to correctly identify and distinguish between arterioles and venules. These identifications were executed by two experienced scientists who had to reach consensus on the vessel type before categorisation and selection (9). The individual data points were exported from Visualis automated software and the estimates were calculated from the 6 largest

arterioles and venules (summarized as the central retinal arteriole equivalent (CRAE) and central retinal venular equivalent (CRVE) respectively) via Excel. These calculations were based on a revised version of the Parr-Hubbard equations, namely the Knudtson "big-six" formulae (10). The imaging scale of each eye differs; thus the values of the CRAE and CRVE were expressed in measuring units (MU), and each 1MU is equivalent to 1µm. Here each 1MU was equivalent to 1µm, when the dimensions of the eye under examination correspond with those depicted by the normal Gullstrand eye (Figure in Chapter 1). The arteriolar:venular ratio (AVR) was calculated as CRAE/CRVE. The reproducibility coefficient was 0.84 and intra-class correlation Cronbach's alpha-reliability index for the arteriolar:venular ratio (AVR) was 0.91 for a randomly selected cohort (9). As determined by Malan and colleagues, the intra-class correlation analysis comprised a mixed-model framework, where random effects were assumed for all subjects and fixed effects were assumed for graders (9).

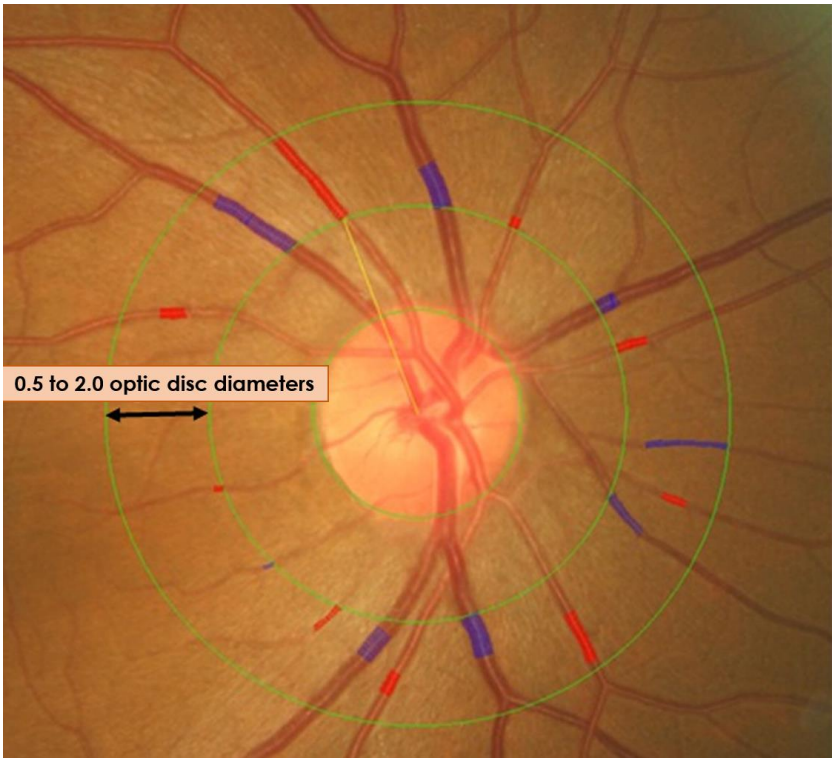


Figure S5: First-order vessel branches as selected within 0.5 and 1 optic disc diameters from the margin of the optic disc

Diastolic Ocular Perfusion Pressure

One to two drops of local anaesthetic (Novasine Wander 0.4% Norvatis) was placed in both eyes prior to the measurement of the intra-ocular pressure (IOP) with the Tono-Pen Avia Applanation Tonometer (Reichert 7-0908 ISO 9001, New York, USA). The diastolic ocular perfusion pressure (DOPP) was calculated by subtracting the IOP from the DBP measured prior to the retinal vessel analyses (Pre-FLIP DBP – IOP mmHg. The DOPP of the right eye was used, as the right eye was used for all retinal vascular measurements (9).

Defining hypertensive/diabetic retinopathy

Diabetic retinopathy was established by a registered ophthalmologist at varying degrees of retinal abnormalities including arteriolar narrowing, arterio-venous nicking (AV-nicking), retinal micro-aneurysms, exudates (hard or yellow exudates), haemorrhages and venous alterations (11). Hypertensive retinopathy was characterized by a range of retinal vascular signs, such as focal or generalized arteriolar narrowing, AV nicking, retinal haemorrhages, micro-aneurysms, cotton-wool spots, hard exudates and an increased optic nerve cup-to-disk ratio (indicative of optic nerve degeneration) – specifically in individuals with hypertensive BP values (11).

Stroke-risk indicators

The 10-year University of California Los Angelis (UCLA) risk composite score included age, gender, SBP, hypertensive drugs, diabetes, smoking habit, perfusion deficits, atrial fibrillation and electrocardiography (ECG) left ventricular hypertrophy (American Heart and Stroke certified UCLA Medical Centre, Primary Stroke Centre, Santa Monica, Los Angeles, USA). Medium to high stroke probability was termed as scores of 5.2 and higher.

Heart-rate-variability calculations

Blood pressure and Heart-rate-variability analyses during FLIP

Event BP (Cardiotens CE120® (Meditech, Budapest, Hungary)) was done at both pre- and post-FLIP phases. The cuffed arm was relaxed and positioned along the side of the participant. Standard BP protocol was followed. HRV measures, time and frequency domain analyses were obtained with the Cardiotens® (Meditech CE120®; Meditech, Budapest, Hungary) and calculated using the CardioVisions® (Meditech CE120®; Meditech, Budapest, Hungary) software. The FLIP response window was viewed for each participant and a minimum window of 15 minutes was selected, containing approximately 33 000 RR-interval data-points per participant. This window was selected to include FLIP and post-FLIP analyses and is referred to as dynamic vessel analyses (DVA)-FLIP. The software removed all arrhythmias and extra-ventricular beats, and additional outliers were manually removed before frequency and time domain variables were calculated for the selected window (Please refer to **Figure S6**). Variables used included time domain parameters, standard deviation of the NN intervals (SDNN), the root-mean squared of the standard deviations of successive RR-intervals (rMSSD) as well as the triangular index (HRVti). These variables were discussed in *Chapter 1*. The HRVti is an index of the pulse variability based on a triangular interpolation method in the given time interval. The histogram assesses the relationship between the total number of RR intervals detected and the RR interval variation. The triangular HRV index

considers the major peak of the histogram as a triangle with its baseline width corresponding with the amount of RR interval variability, its height corresponds with the most frequently observed duration of RR intervals, and its area corresponds with the total number of all RR intervals used to construct it. NN-intervals refer to the intervals between **normal** R-R peaks. During a measurement, artefacts may arise due to arrhythmic events or sensor errors (12). This may lead to abnormal R-R peaks, which may lead to incorrect statistical calculations. To ensure reliability and validity of data, only normal R-R peaks are selected. Please refer to the methods diagram on how the HRV parameters were calculated (**Figure S6**). In practice, however, RR-intervals and NN-intervals are synonymous. The use of "NN-intervals" is to emphasize the normal R-R peaks were used. Frequency domain parameters included the normalized units (nu) of the low frequency bands (LFnu), high frequency bands (HFnu), and the LF/HF ratio. Frequency domain analyses, over shorter periods of time, are more often employed as parameters as these are better equipped to discriminate between the contributions of SNS and parasympathetic (PNS), since they manifest in two overlapping frequency bands. The time domain parameters selected (specifically SDNN and rMSSD) are also used to investigate recordings of short durations (1, 12). It is important to note that components of HRV provide measurement of the degree of autonomic modulations rather than the level of ANS tone (12). Hence for the sake of clarity, all references to time-domain parameters will be described in terms of SNS *activity* and references to frequency domain parameters will indicate SNS *modulation*. Please refer to **Figures S7a and S7b** for a visual representation of how the HRV time- and frequency-domain parameters are derived.

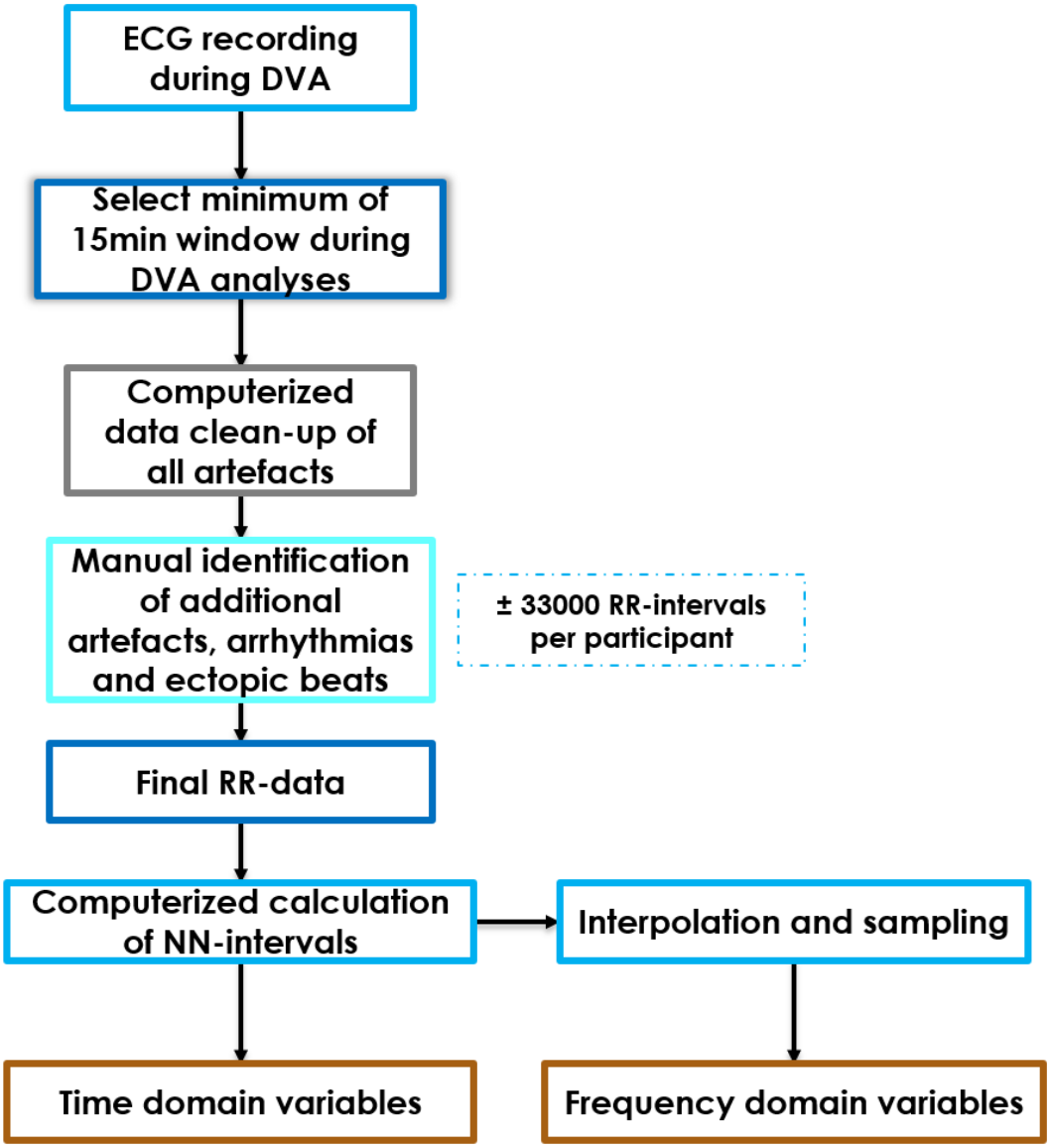


Figure S6: Diagrammatic representation of the procedure followed to determine the HRV parameters during FLIP

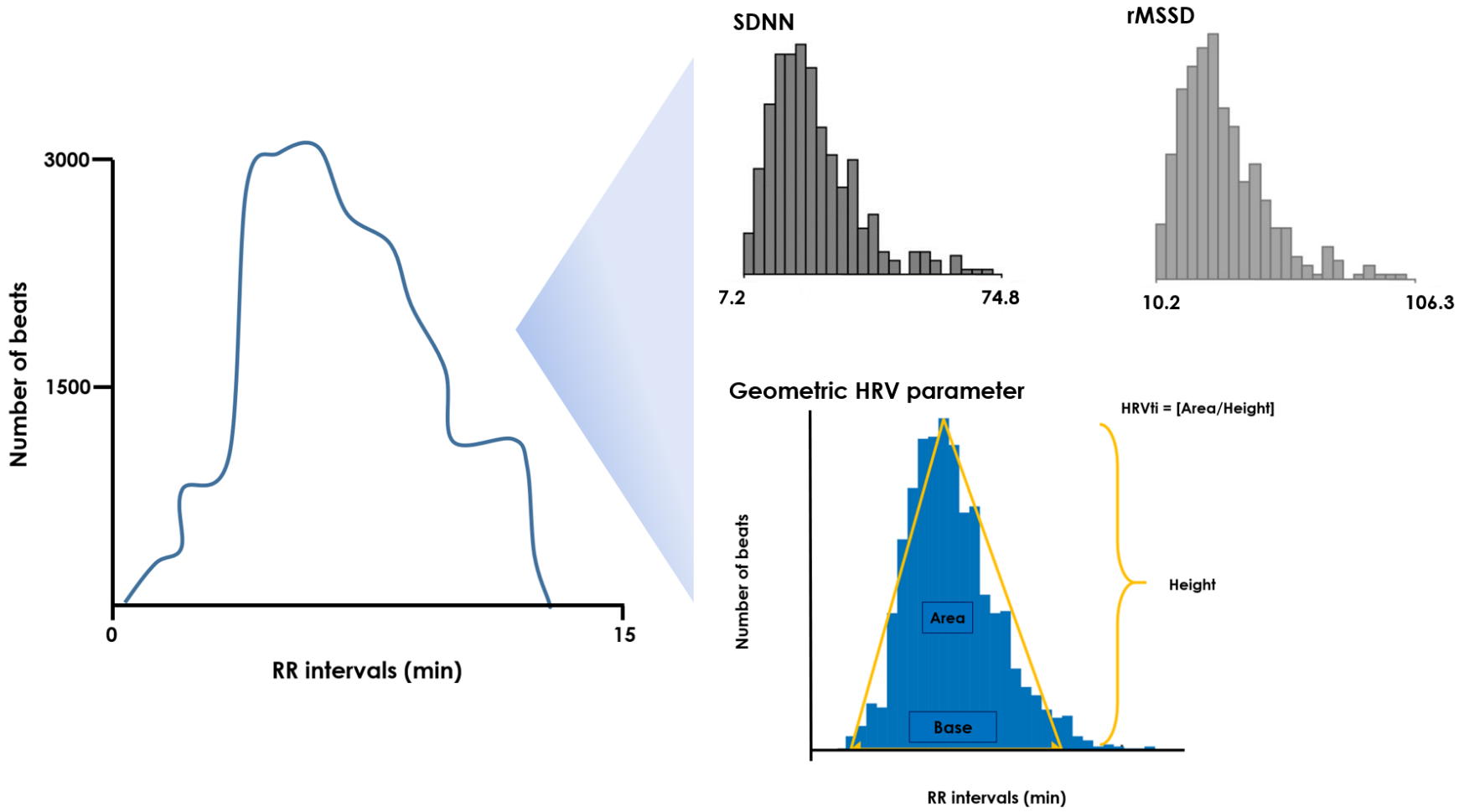


Figure S7a : Derivation of HRV Time-domain variables, standard-deviation of the NN intervals (SDNN), root-mean-squared of the standard deviation (rMSSD) and the geographical calculation of HRV triangular index. Where HRV, heart rate variability

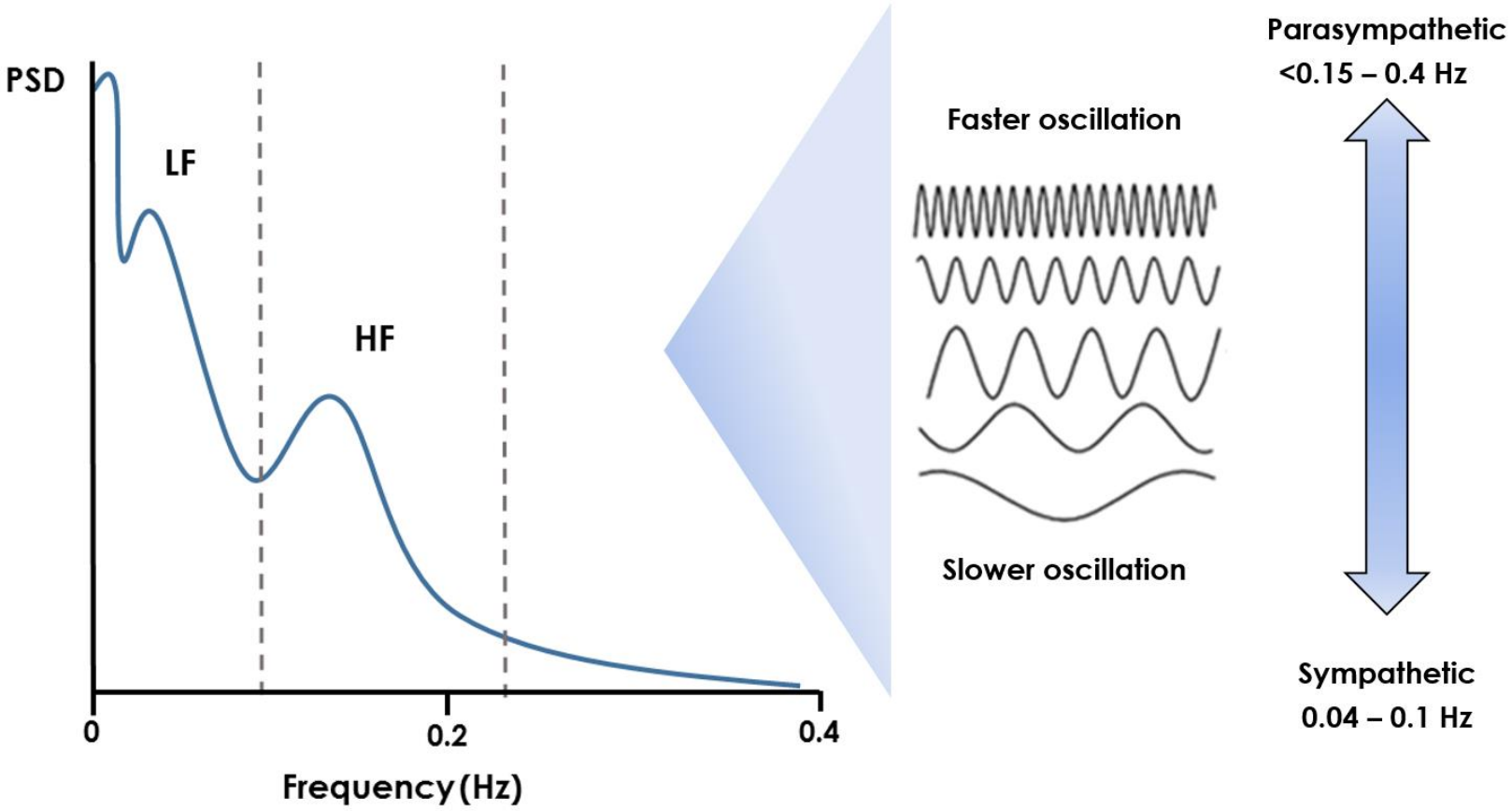


Figure S7b: Derivation of HRV frequency domain parameters, low frequency (LF) and high frequency (HF). Where HRV, heart rate variability; PSD, power spectral density

Biochemical analyses

A registered nurse obtained fasting blood samples were obtained from the antebrachial vein branches of each participant's dominant arm with a sterile winged infusion set. Blood samples were dealt with in accordance with the standardised protocol and serum and plasma samples were frozen at -80˚C until analysed in duplicate.

Cardiac troponin T (cTnT) was measured via an electrochemiluminecence method on the Roche® e411 (Roche®, Basel, Switzerland). Inter-batch variability 15%; intra-batch variability 5.6%. Below detectable limit cTnT values (31.3% of all cTnT analyses, N=104), were logarithmically calculated according to a previously developed method by Crognan and Edgegy (13). The diagnostic accuracy of single baseline measurement of the Elecsys© Troponin T high-sensitivity assay was determined by Zhelev et al. (14). N-terminal pro-B-type natriuretic peptide (NT-proBNP) was also measured via an electrochemiluminecence method on the Roche® Elecsys e411 (Roche®, Basel, Switzerland). Inter-batch variability 4.6%; intra-batch variability 4.2%.

Serum brain-derived neurotrophic factor (BDNF) in ng/ml was determined via a quantikine colorometric-sandwich immunoassay from R&D systems, Minneapolis, MN, USA (Catalogue #: DBD00). Samples were allowed to clot for 30 minutes in a serum separator tube before centrifugation, for 15 at 1000g. The intra-assay and inter-assay precision for SABPA phases 1 and 2 were 3.8-6.2% and 7.6-11.3% respectively.

To determine the presence of a smoking habit, the nicotine metabolite, cotinine (ng/mL), was determined by means of a homogeneous immunoassay with a Modular Roche® automized (Switzerland). Where values were <0.00, 0.01 was added into the dataset to avoid null-values.

The hepatic enzyme gamma glutamyl transferase (γGT)(U/L), associated with alcohol consumption, independent of fatty liver disease or liver failure (15), was measured via an enzyme-rate method with the Unicel DXC800® (Bechman and Coulter®, Germany).

Ultra-high sensitivity C reactive protein (hs-CRP) (mg/L) was analysed by applying a turbidometric method on the Unicel DXC800® (Bechman and Coulter®, Germany). Quantikine high sensitivity tumour necrotic factor alpha, was measured via immune-assay by the accredited laboratory AMPATH (inter-batch variability 15%, intra-batch variability 17.8%). Glycated haemoglobin (HbA_{1c}) EDTA Whole blood HbA_{1C} target levels for diabetic glycaemic control: ideal <7%. Levels at or beyond 6.5% are enough to make a diagnosis of diabetes, while levels from 5.7-6.4% are high risks for developing diabetes and CVD, which is a marker of a pre-diabetic status. (Standards of Medical Care in Diabetes, Diabetes Care, 2010 (American Diabetes Association) HbA_{1C} reference: 4.0 - 6.0 %. Each 1% in change in HbA1c relates to a 2 mmol/l change in p-glucose. Method:

Turbidometric inhibition immunoassay. Apparatus for analysis: Integra 400, Roche, Switserland. High density lipoprotein (HDL) and were determined via a homogeneous enzymatic colorimetric assay, Cobas Integra 400 plus, Roche®, Basel, Switzerland. Total serum cholesterol (mmol/L) was determined via the timed-end-point method, Unicel DXC 800 - Beckman and Coulter, Germany. Thyroxine and thyroxine-stimulating hormone (TSH) were measured via the DRI® Thyroxine and DRI® TSH homogenous enzyme immunoassays respectively (Thermo Scientific, Germany).

Statistical analyses (Figure S8)

Statistica version 13.3 (TIBCO Software Inc., Palo Alto, USA, 2018) was used for data analyses. Kolmogorov-Smirnov tests assessed normality of all variables. Logarithmically transformed γGT, CRP, cotinine and HbA_{1c} levels were used in correlation models. Characteristics between ethnic groups were calculated with *t*-tests. Chi-square (X^2) statistics were used to determine proportions and prevalence data. *A priori* covariates included age, body surface area (BSA), cotinine, γGT, HbA_{1c}, total cholesterol/HDL cholesterol ratio, TNF-α, hypertensive/diabetic retinopathy and 24-hour pulse pressure. Single two-way ANCOVAs determined ethnic x gender differences for all cardiac stress and retinal vessel markers, independent of *a priori* selected co-variates. One-way ANCOVA was used to determine the least square mean difference in response markers between ethnic groups, independent of *a priori* selected covariates. Multivariate linear regression analyses were used to determine associations between main variables in several models as shown in **Figure S8**. For all the aforementioned analyses, significance was set at $p<0.05$ (two-tailed) and the F to enter was fixed at 2.5 in regression models.

Sensitivity analyses

Forward stepwise regression analyses with the same set of covariates were repeated in several models in both ethnic groups for each manuscript.

Analyses were computed by excluding participants with diabetes (n= 36) and those using angiotensin converting enzyme inhibitors (n= 52). None of these variables statistically significantly ($p<0.05$) influenced the outcome (*Manuscript 1*).

Forward stepwise regression analyses, with the same set of covariates, were repeated in several models in both ethnic groups. Excluding participants with diabetes (n=78), on diabetic treatment (n=29) and those using any form of hypertensive treatment (n=76), did not influence the outcome (*Manuscript 2*).

Forward stepwise regression analyses, with the same set of covariates, were repeated in several models in both ethnic groups. Excluding participants using any form of hypertensive treatment (n=81), did not influence the outcome (*Manuscript 3*).

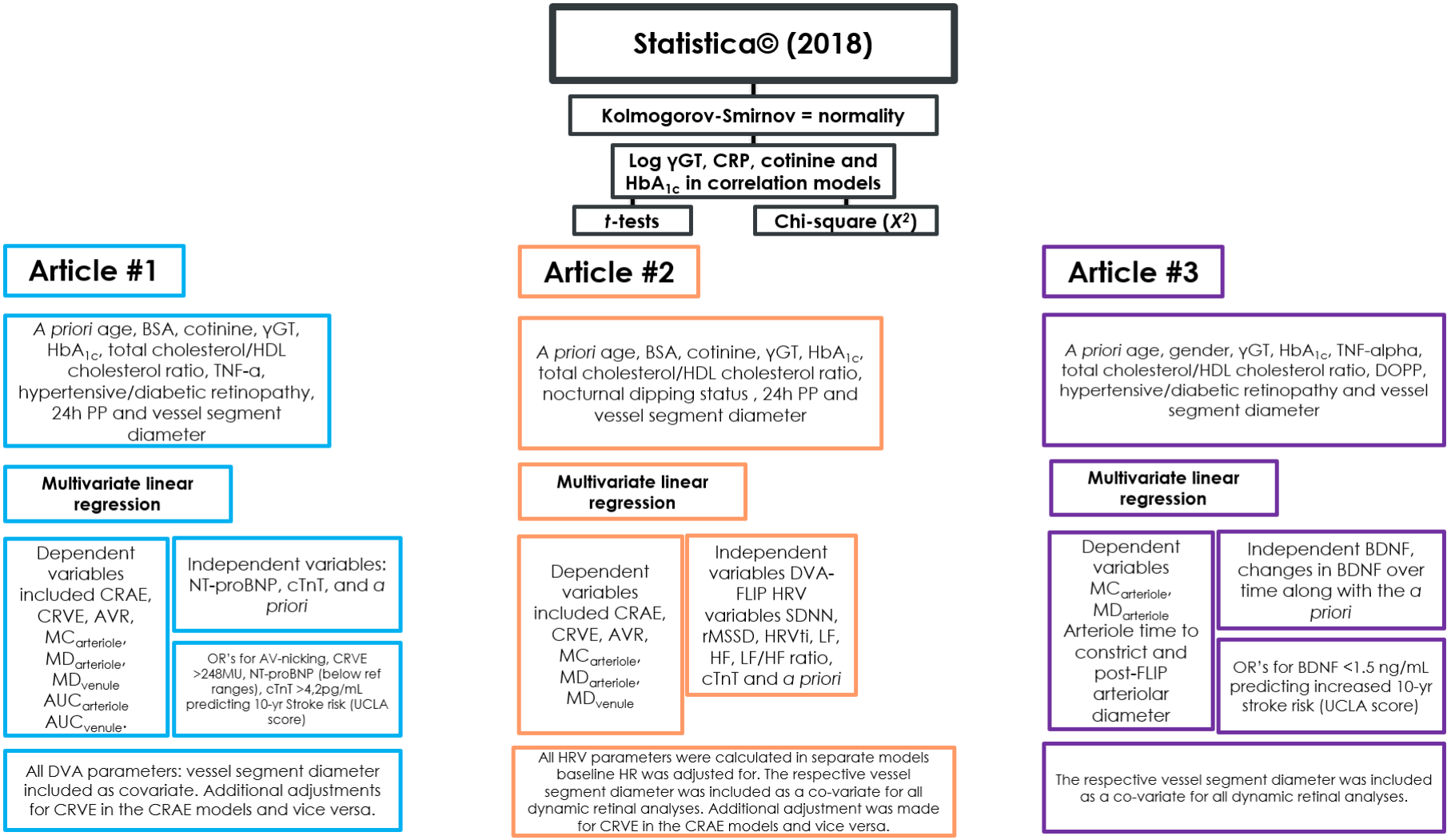


Figure S8: Graphical representation of the statistical analytical procedure followed for each manuscript/ article.

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Glossary

***Here follows the explanation of some terms/definitions/concepts/notions/expressions/phrases/labels frequently applied within the context of this thesis**

Amino-terminal pro-B-type natriuretic peptide (NT-proBNP) – All natriuretic peptides are synthesized as pre-hormones and subsequently cleaved by multiple enzymes to become biologically active. proBNP is released in response to ventricular volume expansion and/or pressure overload, and its N-terminal rapidly enzymatically cleaved to obtain biologically active BNP. B-type natriuretic peptide (BNP) is a ringed peptide secreted by the heart and brain to regulate fluid balance and blood pressure.

Arteriolar Narrowing – When the arteriolar diameter decreases, causing a narrower arteriole to be observed. Common, characteristic sign in hypertension and associated with stroke risk.

Attenuated Retinal Vessel Responses – When a poor, adverse, undesirable or pathological retinal vessel reaction is observed during a stressor (such as flicker light). Usually seen as weaker arteriolar constriction, dilation, delayed reaction times or prolonged vessel responses.

Autonomic Nervous System (ANS) – The division of the nervous system responsible for regulating involuntary bodily functions including the activity of the cardiac muscles, smooth muscles and glands – usually in response to external/ internal stimuli. The ANS is sub-divided into the **sympathetic nervous system (SNS)**, mainly responsible for accelerating heart rate, constricting blood vessels and increasing blood pressure. Contrarily the **parasympathetic nervous system (PNS)** slows the heart rate, increases intestinal peristalsis, relaxes sphincters and increases gland activity.

Autonomic Nervous System (ANS) dysregulation – ANS dysregulation may develop in severe cases when the nerves of the ANS are damaged (ANS neuropathy or dysautonomia). Dysregulation of the ANS may also imply that one leg, usually the sympathetic component, exerts a greater effect than the parasympathetic component, leading to an imbalanced ANS function and drive.

Autoregulation – The inherent ability of microvascular systems (including the retinal, cerebral and coronary microvasculature) to maintain a relatively constant blood flow despite varying arterial/ systemic blood pressure. This ability results from an internal adaptive mechanism that adjusts a systems' response to a particular stimuli.

AV-nicking – The phenomenon where an arteriole crosses a venule, resulting in the compression of the venule, observed with bulging on either side of this crossing. A common occurrence when high systemic blood pressure is present and forms part of the diagnostic criteria of hypertensive retinopathy.

Bayliss-effect – The vascular smooth muscles' response to stretch. The Bayliss effect is a special manifestation of myogenic tone in the vasculature whereby the arterioles respond to changes in systemic blood pressure. When systemic blood pressure increases, the blood vessels distend, and the arteriolar vascular smooth muscle cells respond with constriction, ensuring adequate and constant perfusion pressures despite systemic blood pressure fluctuations.

Blood-brain Barrier – A highly selective, semipermeable physiological border that separates the peripheral circulating blood from the brain and CNS. The blood-brain barrier is comprised of unique blood vessels, which allow for the tightly regulated movement of ions, molecules and biological components between the blood and the brain. The components that make up this barrier coordinate by way of physical, metabolic and transport properties and include the endothelial cells of the blood vessels, astrocyte end-feet (surrounding capillaries and arterioles) as well as pericytes that are embedded in the basement membrane of capillaries and arterioles.

Blood-retina Barrier – Similar to that of the blood-brain barrier, the blood-retina barrier is a highly intrinsic barrier system composed of both an inner and outer barrier. The outer blood-retina barrier is mainly formed by the retinal pigment epithelial cell layer and regulates the movement of solutes, nutrients and biomolecules from the choroid to the sub-retinal space. The inner blood-retina barrier is similar to the blood-brain barrier and mainly comprise of the retinal microvasculature endothelium, tight junctions, astrocytic end-feet and pericytes.

Cardio-metabolic Risk – Cardio-metabolic concerns both heart and metabolic disorders such as diabetes and hypertension. The cardio-metabolic risk is a condition in which the probability of developing atherosclerotic and diabetes mellitus are significantly higher compared to controls. Patients diagnosed with metabolic syndrome present with a particularly high cardio-metabolic risk.

Cardiac Stress – When there is increased strain on the cardiac muscle, finally resulting in cardiomyocyte distress or damage. It is usually the consequence of increased pressure and/or volume loading conditions. The biomarkers cTnT and NT-proBNP are usually measured to determine the degree of cardiac stress within epidemiological as well as clinical settings.

Cardiac Troponin T (cTnT) – Cardiac troponin T (cTnT) forms part of the cardiac troponin complex. cTnT is a cardiac regulatory protein that controls the calcium mediated interaction between actin and myosin.

Depressed HRV – Indicates a poorer vagal influence (vagal withdrawal) and increased SNS influence.

Dynamic Vessel Analyses (DVA) – A relatively new method that allows for the online measurement of retinal vessel calibres and changes therein during flicker light-induced provocation. This allows

for the analyses of reactivity in the retinal microvasculature and provides a unique window to non-invasively study the condition of the microvasculature and endothelial function elsewhere in the body.

Flicker Light Induced Provocation (FLIP) – The stimuli applied in order to assess dynamic changes in retinal vessel diameters. It consists of three consecutive periods of monochromatic flicker-light stimulation (530–600 nm, 12.5 Hz, 20 s).

Glial cells – The supporting, non-neural cells of the central and peripheral nervous systems. Glia are responsible for maintaining homeostasis in the central and peripheral nervous systems, providing structural and metabolic support to neurons. Types of neuroglia include astrocytes, microglia and oligodendrocytes.

Heart Rate Variability (HRV) – Heart-Rate-Variability (HRV) is the most often employed non-invasive assessment of ANS tone and modulation. HRV is the variation in the time interval between successive heart beats and indicates neuro-cardiac function, generated as a result of brain-heart interactions and dynamic, non-linear ANS processes. The time-domain parameters of HRV, specifically the standard-deviation of the NN-interval, are considered the gold standard for reporting SNS activity. The frequency-domain parameters are employed for shorted recordings, and reflect SNS modulation.

Hemodynamics – Also termed the dynamics of blood flow which explain the physical laws that govern the flow of blood in blood vessels. As blood is a non-Newtonian fluid, rheology (the study of the flow of matter in liquid state), rather than classic hydrodynamics, are used to describe blood flow and changes therein.

Hyperaemia – Increase of blood flow to specific tissue areas. Functional and reactive hyperaemia have been defined. ***Functional hyperaemia*** (also known as active hyperaemia, metabolic hyperaemia or arterial hyperaemia) is when an increase in blood flow to a target area is prompted by the presence of metabolites due to increased activity of said tissue. ***Reactive hyperaemia*** (also termed venous hyperaemia) is the transient increases in tissue blood flow that occurs after a brief period of ischemia.

Ischemic stroke risk – Stroke is an acute neurological deficit that may persist over 24 hours and is due to cerebrovascular etiology. Ischemic stroke is a subdivision of stroke, and is traditionally caused by vascular occlusion or stenosis. Transient ischemic episodes or attacks are defined as transient episodes of neurological dysfunction due to focal brain, spinal cord or retinal ischemia, with the absence of an acute infarction. However, patients with such transient ischemic episodes are at a greater risk for ischemic stroke, and such risk may be stratified using retinal vessel imaging.

Myogenic tone – The muscle tone that is ascribed to the muscle itself, independent of the ANS or hormonal processes. Myogenic tone is one of the contributors to the myogenic mechanism, which is the mechanism by which the microvasculature (arterioles and to a lesser extent venules) react to changes in systemic blood pressure, to ensure constant local perfusion pressures (*Please see Bayliss effect*).

Neurotrophic Growth Factors – A family of peptides that support the growth, survival and differentiation of both developing and mature neurons. In the mature nervous system, they promote neuronal survival, synaptic plasticity and maintenance of blood-brain and blood-retinal barriers. Neurotrophic factors usually exact their trophic effects via tyrosine kinase signalling or TrkB receptors. The main family of neurotrophic factors of interest in this study is the neurotrophins, which include **brain-derived neurotrophic factor (BDNF)**. BDNF plays important regulatory roles in the maintenance of the blood-retinal barrier, development of the visual cortex, neurogenesis, learning and memory formation.

NN-interval – NN-intervals refer to the intervals between normal R-R peaks. During a measurement, artefacts may arise due to arrhythmic events or sensor errors. This may lead to abnormal R-R peaks, which may lead to incorrect statistical calculations. To ensure reliability and validity of data, only normal R-R peaks are selected. In practice, however, RR-intervals and NN-intervals are synonymous. The use of "NN-intervals" is to emphasize the normal R-R peaks were used.

Rarefaction – A reduction in the density of blood vessels (reduced number of arterioles and venules per square area). Usually the result of sustained high pressure, causing defective oxygen and nutrient delivery resulting in death – rarefaction – of the terminal arterioles and venules.

Retinal Neurovascular Coupling – Neurovascular coupling refers to the relationship between local neural activity and the subsequent changes in retinal blood flow. The magnitude and spatial location of blood flow changes are tightly linked to changes in neural activity via a complex sequence of coordinated events involving neurons, glia and vascular cells.

Retinal Vasculature Reactivity – Changes that occur in retinal vessel diameters during application of a stressor (flicker light), including arteriolar constriction, dilation as well as venular dilation.

Retinopathy – A group of eye disorders (usually inflammatory in nature). The most common contributing conditions include hypertension, diabetes, atherosclerotic and arteriosclerotic vascular diseases.

Stressor – Any biological, chemical, environmental or external stimulus that triggers the stress response. Psycho-physiologically it can also be any event or environmental changes that an individual may consider demanding, threatening or challenging. Within the context of this thesis, the

flicker light (FLIP) is deemed, and newly defined as a SNS stressor, which not only elicits biomechanical and metabolic responses, but also SNS driven responses.

Sympathetic Nervous System (SNS) hyperactivity – When there is excessive activity in the SNS leg of the autonomic nervous system. This results in increased blood pressure and heart rate which may result in long term perfusion deficits.

Vascular dysfunction – Defined as structural impairment of the microvasculature that may lead to further functional impairment, thereby affecting the vasculature's ability to dilate and contrict.

Vascular dysregulation – Usually defined as a functional impairment which may precede any structural alterations, where there is attenuated dilation or constriction without permanent structural modifications.

Venular Widening – When the venular diameter increases, causing a wider venule to be observed. Associated with increased stroke risk, and evident in volume loading hypertension.

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Dr L Malan

Dear Dr Malan

6 February 2008

ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title:.SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans)

***Ethics* number**: N W U - 0 0 0 3 6 - 0 7 - S 6
(Institution | Project Number | Year | Status)
Status: S = Submission, R = Re-Submission, P = Provisional Authorisation, A = Authorisation

Approval date: 12 November 2007 **Expiry date**: 11 November 2012

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-EC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-EC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof M M J Lowes
(chair NWU Ethics Committee)

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To whom it may concern

31 August 2012

Dear Prof./Dr./Mr./Me.

Ethics application: NWU-00036-07-S6 (L. Malan)

"SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans)" study

The additional request for continuation of the SABPA studie till 2017 has been approved.

Kind regards

Prof. H.H. Vorster
Chair person

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Prof L Malan
HART

30 September 2018

Dear Prof Malan

FEEDBACK ON HREC ANNUAL MONITORING REPORT: NWU-00036-07-A6

We would like to thank you for submitting the annual monitoring report for your project entitled, ***"The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study"***, to the Health Research Ethics Committee (HREC) in a timely manner. Please find below the decision of the HREC committee regarding the continuation of your project.

Classification	Mark with X	Comment	
Clarification			
Completion (Final report)			
Suspended			
Continuation	X	**Date of next monitoring report:**	30 September 2019
Termination			

Should you have any further queries, please feel free to contact Ms Jamey Henry at your earliest convenience (E-mail: Ethics-HRECMonitoring@nwu.ac.za; Tel: 018 299 2266). We wish you well in your future endeavours.

Yours sincerely

Prof Minrie Greeff
Head of Health Sciences Ethics
Office for Research, Training and Support

Prof Wayne Towers
Chairperson: HREC

Current details: (20536690) G:\My Drive\9. Research and Postgraduate Education\9.1.5.5 HREC Monitoring\NWU-00036-07-A6\9.1.5.5.4_Cont_NWU-00036-07-A6_30-09-2018.docm
30 September 2018

File reference: 9.1.5.5.4

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Email : Ethics@nwu.ac.za

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **Health Research Ethics Committee (HREC)** on **13/10/2017**, the North-West University Research Ethics Regulatory Committee (NWU-RERC) hereby **approves** your study as indicated below. This implies that the NWU-RERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Neural control and retinal vascular dynamics' relation to cardiac stress: The SABPA study

Study Leader/Supervisor: Prof L Malan

Student: A Wentzel-23615109

Ethics number:

N	W	U	-	0	0	0	5	7	-	1	7	-	A	1
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study

Commencement date: 13/10/2017 **Risk:** Minimal

Approval of the study is initially provided for a year, after which continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The study leader (principle investigator) must report in the prescribed format to the NWU-RERC via HREC:*
  - *annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-RERC and HREC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the HREC or that information has been false or misrepresented,*
    - *the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *HREC can be contacted for further information or any report templates via Ethics-HRECApply@nwu.ac.za or 018 299 1206.*

The RERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the RERC or HREC for any further enquiries or requests for assistance.

Yours sincerely,

Prof Refilwe Phaswana-Mafuya
Chair NWU Research Ethics Regulatory Committee (RERC)

PARTICIPANT INFORMATION AND CONSENT FORM

NORTH-WEST UNIVERSITY
POTCHEFSTROOM CAMPUS

SCHOOL FOR PHYSIOLOGY, NUTRITION AND CONSUMER SCIENCES

PARTICIPANT INFORMATION AND CONSENT FORM

PART 1

PRINCIPAL RESEARCHER: Prof Leoné Malan, Subject Group Physiology

PROJECT LEADER: Prof Leoné Malan

Associate Researcher(s): The postdoctoral fellows involved in this trial are Dr. P Szabolcs, Mr M Glynn. Other persons assisting in the study are Proff Nico T Malan, Alta E Schutte, Hugo W. Huisman, Johannes M. van Rooyen, Rudolph Schutte, Drs. Carla M.T. Fourie, Wayne Smith, Carina Mels, Mrs. Tina Scholtz, Lisa Uys, Mr Ruan Kruger (Hypertension in Africa Researc Team), Proff. Hans de Ridder (Anthropometry, Physical activity), Johan Potgieter, Dr Tumi Khumalo (Psychology), Proff Linda Brand & Brian Harvey (Pharmacology), Kobus Mentz (Education), Francois van der Westhuizen (Biochemistry), Ronel Pretorius (Nursing), Yackoob Seedat (Kwazulu Natal), Paul Rheeder (Pretoria University), Proff Nancy Frasure- Smith & Francois Lespérance (Canada), Drs Alaa Alkerwi (Luxembourg), M Hamer (UK), Manja Reimann, Proff Tjalf Ziemssen, C Kirschbaum (Germany), Eco JCN de Geus (Netherlands); Markus Schlaich & Dr G Lambert (Australia), Prof Morten Rostrup (Norway).

This Participant Information and Consent Form are **8** pages long. Please make sure you have all the pages.

Your Consent

You are invited to take part voluntarily in this research project.

This participant information document contains detailed information about the research project which has been explained to you verbally. Its purpose is to explain to you as openly and clearly as possible all the procedures involved in this project before you

decide whether or not to take part.

Please read this *Participant Information Form* carefully. Feel free to ask questions about any information in the document. You may also wish to discuss the project with a relative or friend or your local health worker. Feel free to do this.

Once you understand what the project is about and if you agree to take part in it, you will be asked to sign the Consent Form. By signing the Consent Form, you indicate that you understand the information and that you give your consent to participate in the research project.

You will be given a copy of the Participant Information and Consent Form to keep as a record.

What is the study about?

The aim of this project is to have an impact on the eventual prevention and treatment of lifestyle diseases in Africans from South Africa. New knowledge regarding the relationship between higher nervous system activity implicating cardiovascular, metabolic and psychological well-being will improve understanding and change strategies at the roots of treatment and prevention of lifestyle diseases.

Our research has shown that lifestyle diseases in urban Africans present higher obesity levels, high blood pressure or hypertension prevalence rates and the experiencing of more stress. This pattern is enhanced during psychosocial stress/urbanisation in participants with a specific coping style. Hence, the planned SABPA project, which is the first study in South Africa where coping and direct markers of in Africans will be measured.

Purpose of study

The purpose of this study is to ***repeat most of our previous measurements*** although not the stressor exposure measures. We will investigate biological markers associated with higher nervous system activity in urban teachers with a specific coping style.

To investigate the relationship between blood pressure, inflammation, obesity, stress and coping in more detail we are going to perform this study in 409 men and women from the North-West province, aged 25-65 years. A comprehensive assessment of the cardiovascular and nervous systems by means of non-invasive painless techniques will be performed and blood and saliva samples will be taken by an experienced research nurse to determine your blood sugar, cardiovascular, inflammation and stress hormone levels amongst other health markers.

Procedures

All measurements are performed in the Metabolic Unit (lipid clinic) of the University. A researcher has explained the entire procedure in detail and while you are reading this information document you have time to ask questions and to have clarified matters. If you are fine with the explained procedure you are requested to sign a *consent form (at the end of this document). Remember all personal data will be handled with care and remain confidential.

**By consenting to participate in this study, you consent to the storage and later analysis and testing of your stored blood samples for the purposes noted above. Your blood will also be tested for preliminary results on HIV status, since your HIV status may directly influence the main purposes of this study. If you would like to know what your HIV-status is, we will provide it. If tested positive we will refer you to your doctor and he/she will perform the necessary tests which will allow you to apply for chronic medication benefits. Also, the blood cells from your donated blood sample will be*

used to investigate the molecular genetics of higher nervous system activity and Type 2 diabetes in order to enable pre-symptomatic diagnosis of hypertension and diabetes in the long term.

Why was I chosen? Educators are exposed to changing curricula and disciplinary problems whilst living in an urban environment adding to higher stress experiencing and nervous system activity.

How was I chosen?

Inclusion criteria*:*
All SABPA I (2008/2009) black (Phase I) and Caucasian (Phase II) teachers (male and female)

Exclusion criteria: *pregnancy, lactation, temperature >37°C.* ***You can not be included if you have donated blood or been vaccinated in the previous 3 months****.*

What will be expected of me?

You, as participant will be screened once by a registered nurse to be eligible complying with the inclusion criteria. The following procedures will be followed:

- Recruitment and informed sessions with all participants will be done two months prior to the study (October - November 2010, Phase I, and **November, 2011, Phase II**) and informed consent forms will be signed.
- After selection of all participants, the details of the project will be discussed with you in English or your home language, i.e. what the exact objectives of the study are, what procedures will be taken and what will be expected from each of you (e.g. overnight stay, resting blood pressure procedures and fasting urine and blood samples are required, importance of complying with the correct sampling methods, incentives). You will be given the opportunity to ask questions.
- Data collection for each participant will involve two days (15 min in the morning and 2½ hours in the evening) on Day I; and 2 hours on Day II):

DAY I

- On day I between 07:00-08:00, the blood pressure apparatus, which will measure your blood pressure and heart function will be applied to your arm and waist at your school and you can then resume your normal daily activities.
- Urine sampling (24h) and 24h diets will commence.
- At the end of Day I (**15:00**) you have to visit us or be transported from your schools to the Physiology F12 building (NWU) and will overnight in the Metabolic Unit Research Facility of the North-West University. This unit is a research unit for human studies and equipped with 10 well furnished bedrooms, a kitchen, two bathrooms and a television room. Each of you will be subjected to the following procedures:
  - At 15:00 you will be welcomed at F12 at the HART clinic and eye measurements including saliva sampling pertaining to cardiometabolic health will commence.
  - Hair sampling and pre-counselling for HIV/AIDS will be done.

- You will go then go the Metabolic Unit Research Facility of the North-West University (G17) to receive your own bedroom. All other apparatus will be shown and the procedures, which will be done, will be explained again and you will receive dinner.
- After dinner, the psychosocial questionnaires will be completed under supervision of registered clinical psychologists/postgraduate students. Completion of questionnaires will take approximately 40 min, From 22:00 you will be fasting, therefore, this will be you last meal for Day I as you must be fasting on Day II for obtaining good results.
- Thereafter, you can relax and watch television or socialize with your co-participants. It will be wise to go to bed not later than 22:00 as the blood pressure apparatus will take measurements every hour during the night and it can be tiring.

DAY II

- At 06:45 on Day II in the anthropometric station your weight, height and body circumferences will be measured.
- Urine sampling will be completed before 07:30 whereafter the blood pressure apparatus will be removed (07:30 after last measurement).
- Next the cardiovascular measurements will follow consisting of three separate procedures:
  - Firstly, after being in semi-recumbent position for 10 minutes your blood pressure will be taken in duplicate with the sphygmomanometer (the same as used at clinics) with a resting period of 5 minutes in between.
  - Secondly, our registered research nurse will measure the ECG which measures heart function, with 12 leads, which will be placed into position on your rib cage/front part of the body.
  - Thirdly, the assessment of pulse wave velocity will follow, i.e. giving an indication of how stiff your vessel walls are. The stiffer your vessel wall is the faster the blood travels from one point of your body to another. These painless measurements will require two technicians using blunt probes (tonometer) putting light pressure on the neck and on the foot to measure the velocity of the pulse waves. This takes only a few minutes.
  - A once-off blood sample of 48 ml will be obtained between 08:30 - 09:00 from a vein in your dominant arm.
  - Lastly, an ultrasound device will be taken of your arteries in the neck with a blunt probe to indicate the intrinsic thickness of your arteries which contributes to high blood pressure.
  - You have reached the end of the sampling phase.
- Immediate feedback on your HIV/AIDS status, obesity levels, blood pressure and blood glucose/sugar values will be given. *HIV/AIDS post-test counselling will be arranged if you are tested positive.*
- Thank you for your participation! You now will have the opportunity to shower and a take away breakfast will be given.
- You will now be transported back to your school and after one week you will receive your 24-hour blood pressure, 12 lead ECG and eye reports as well as sleeping disturbances/sleep apnea risk.

Possible Risks

The measurements performed in our study will include only non-invasive techniques that are not expected to reveal any risks but might cause little discomfort. The taking of blood samples is an invasive procedure with a minimal risk of bleeding. Thus the procedure may cause only a few seconds of light discomfort. All tests will be performed by experienced research nurses of our department. There may be additional unforeseen or unknown risks.

Precautions to protect the participant

The Metabolic Unit facility of the NWU is fully equipped, and in case of an emergency which could not be handled by the registered nurse, the supervising medical doctor P de K Geldenhuys will be contacted. Dr. Geldenhhuys was notified before the study commenced that this study will be taking place, and that there is a slight possibility that he may be contacted. Supporting medical treatment care facilities will be at hand anytime if needed.

Other Treatments Whilst on Study

It is important to tell the research staff about any treatments or medications you may be taking, including non-prescription medications, vitamins or herbal remedies during your participation in the study.

Incentives

1. All teachers will receive feedback on their health profile and if necessary references will be given to physicians/clinics/hospitals.
2. Blood pressure, kidney functioning, eye measures and ECG monitoring (normally costing R8000.00). Your benefit of participation is a comprehensive assessment of the cardiovascular and metabolic condition including investigation of blood pressure, inflammatory status and psychological well-being. These examinations will help us to assess the degree of vascular impairment of the arteries and to predict your risk of possible cardiovascular events such as heart attacks and stroke. The results may assist your doctor in decision making for further treatment or for instituting preventive measures. Our study will also contribute to the identification of possible factors leading to high blood pressure. As 24 hour ambulatory blood pressure monitoring is required for the diagnosis of hypertension, medical aids insist on this method of diagnosis to qualify for chronic medication. Additional testing could also reveal illnesses of a chronic nature and would serve as a motivation to qualify for chronic medication, such as metabolic syndrome, anti-inflammatory and

Privacy, Confidentiality and Disclosure of Information

By consenting to participate in this study, you consent to the storage and later

analysis and testing of your stored blood samples for purposes noted above. Your blood samples will be discarded immediately after analysis. All information provided by you and the results of tests will be treated in the strictest confidence, and will only be used for the purpose of this research project. It will only be disclosed with your permission, except as required by law. The results of your medical tests will be labeled only with a code number, and will be stored separately from any identifying information. When the results are analyzed we will be looking for differences between groups of people, not at the results of individuals. No information that could identify any person taking part in the study will be revealed when the results are reported.

Participation is Voluntary

Participation in any research project is voluntary. If you do not wish to take part you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with the North-West University.

Before you make your decision, a member of the research team will be available so that you can ask any questions you have about the research project. You can ask for any information you want. Sign the Consent Form only after you have had a chance to ask your questions and have received satisfactory answers.

If you decide to withdraw from this project, please notify a member of the research team before you withdraw.

Ethical Guidelines

This project will be carried out according to Ethical Guidelines of the Helsinki declaration from 2008, with additional notes in 2002. This statement has been developed to protect the interests of people who agree to participate in human research studies.

The ethical aspects of this research project have been approved by the Human Research Ethics Committee of ***North-West University Potchefstroom: (NEW-EC): 0003603S6***

Further Information or Any Problems

If you require further information or if you have any problems concerning this project, you can contact the principal researcher or *the other* researchers responsible for this project.

Project Leader Prof Leoné Malan (018-299 2438); Cell 0733765321

Signature

PART 2

To the subject signing the consent as in part 3 of this document

You are invited to participate in a research project as described in paragraph 2 of Part 1 of this document. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. Participation in this project is voluntary.

2. **It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.**

3. **You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.**

4. **The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.**

5. **We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.**

PART 3

Consent

Title of the project: "**THE SABPA STUDY** (**S**ympathetic activity and Ambulatory Blood Pressure in Africans)".

I, the undersigned…… (full names) read / listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project.

(Signature of the subject)

Signed at ..... on ……2011/12

Witnesses

1. .....

2. .....

Signed at ..... on ……20011/12

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I, **Ms Cecilia van der Walt**, hereby declare that I took care of the **editing** of the **thesis** of **Ms Annemarie Wentzel** titled ***Neural Control, Cardiac Stress and Retinal Vascular Dynamics: The SABPA Study.***

C. Van der Walt.

MS CECILIA VAN DER WALT

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