Oxidative stress and angiogenesis in Africans and Caucasians: The SAfrEIC study

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Innovation through diversity

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Contribution of the authors

The following researchers contributed to this study:

Ms. CJ Butler

Responsible for literature searches, statistical analyses, processing data, design, planning and writing of the manuscript.

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Supervised the writing of the manuscript, responsible for the design of the study and collection of data, reading through the dissertation, making recommendations and giving professional input.

This is a statement from the co-authors confirming their individual role in the study and giving their permission that the manuscript may form part of this dissertation.

Prof. AE Schutte	**Prof. R Schutte**

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Summary

Motivation and aim

The prevalence of hypertension is higher in Africans compared to Caucasians. African Americans also show higher levels of oxidative stress. However, literature regarding levels of oxidative stress and the angiogenic growth factors, vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2), are limited in black South Africans. There is also a dearth of literature regarding the relationship between oxidative stress and angiogenesis in hypertensive individuals. Therefore, the aim of this study was to investigate whether a relationship exist between the two angiogenic growth factors and oxidative stress and to determine their relationship with cardiovascular measurements.

Methods

This study was a sub-study of the cross-sectional SAfrEIC study (South African study on the influence of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function) and originally included 750 African and Caucasian men and women aged 20 to 70 years. Only 626 participants' information was used after excluding pregnant or lactating women, as well as those infected with the human immunodeficiency virus. The participants were from semi-urban areas in the North West Province of South Africa

Anthropometric measurements of each participant were taken in triplicate following standard procedures. Systolic and diastolic blood pressure and heart rate were measured after a 10-minute rest in a sitting position, using the OMRON HEM-757 device. Two measurements were taken with a 5-minute rest interval. Cardiovascular measurements were performed using the Finometer^{TM} device. Fasting blood glucose was directly measured in the Metabolic Unit by a nurse using an enzymatic method to screen for diabetes mellitus. A fasting blood sample was taken from the antebrachial vein using a sterile winged infusion set and syringes. Standard methods were used to prepare plasma and serum samples, which were stored at -80ºC until analyses. Serum reactive oxygen species (ROS) were determined with a high-throughput

spectrophotometric assay, with 1 unit equalling 1 mg/liter H_2O_2. Human $VEGF_{165}$ and human Ang-2 were determined with enzyme-linked immunosorbant assays (ELISA).

Results

When viewing the characteristics of the African and Caucasian groups, the Africans showed higher blood pressures ($p<0.001$) as well as significantly higher levels of ROS ($p=0.002$), VEGF ($p=0.002$) and Ang-2 ($p<0.001$). They also included more smokers ($p<0.001$) and hypertensive individuals ($p<0.001$). The use of anti-hypertensive medication was significantly higher in the Caucasian group ($p<0.001$).

In single regression analyses, there were no significant correlations between VEGF or Ang-2 and blood pressure in the African and Caucasian groups. ROS correlated significantly with the two angiogenic growth factors in both groups of men, being stronger in the African men (both $r=0.33$; $p<0.001$) but less so in the Caucasian men ($r=0.16$; $p=0.04$ and $r=0.16$; $p=0.07$). ROS was also significantly correlated with Ang-2 in the African women ($r=0.26$; $p=0.003$). In addition to this, ROS associated significantly, but weakly with diastolic blood pressure in the Caucasian women ($r=0.15$; $p=0.03$).

We plotted VEGF and Ang-2 by quartiles of ROS and adjusted for age and body mass index. In all instances, African men and women showed significant associations of VEGF and Ang-2 with ROS (p for trend < 0.05), except for the association between VEGF and ROS in African women (p for trend=0.80). Conversely, no significant associations were indicated for the Caucasian gender groups.

To further investigate the significant associations found only in the African group, we performed multiple regression analyses with blood pressure or markers of angiogenesis as dependent variables. After full adjustment for confounders, the associations of both the angiogenic growth factors with ROS in the African men (both $p=0.014$) and Ang-2 with ROS in the African women ($p=0.025$) were confirmed. No associations were found between the angiogenic growth factors or ROS with blood pressure.

Conclusion

We found in our study, involving angiogenic growth factors and oxidative stress, that Ang-2 was significantly associated with oxidative stress in African men and women. Additionally, VEGF was linked to oxidative stress only in African men. These associations were absent in both the Caucasian gender groups. The strong association found in the African population possibly add to the existing high risk of cardiovascular disease in this population.

Afrikaanse titel: Oksidatiewe stres en angiogenese in swart en wit mense: Die SAfrEIC studie.

Opsomming

Motivering en doelstelling

Swart mense het ‘n hoër voorkoms van hipertensie in vergelyking met wit mense. Swart Amerikaners het ook hoër vlakke van oksidatiewe stres getoon. Literatuur is skaars rakende die vlakke van oksidatiewe stres en die angiogeniese groeifaktore, vaskulêre endoteel groeifaktor (VEGF) and angiopoietin-2 (Ang-2) in swart Suid-Afrikaners. Daar is ook ‘n tekortkoming in literatuur rakende die verhouding tussen oksidatiewe stres en angiogenese in hipertensiewe individue. Na aanleiding hiervan is die doel van díe studie om te ondersoek of ‘n verhouding bestaan tussen die twee angiogeniese groeifaktore en oksidatiewe stres, asook om hul verhouding met kardiovaskulêre metings te bepaal.

Metode

Hierdie studie was ‘n sub-studie van die SAfrEIC studie *(South African study on the influence of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function)* wat ‘n dwars-deursnee studie ontwerp gehad het. Die studie het oorspronklik 750 swart en wit mans en vroue, tussen die ouderdomme 20 tot 70 jaar, ingesluit. Slegs 626 deelnemers se inligting is gebruik nadat swanger en lakterende vroue uitgesluit is. Persone geïnfekteer met die menslike immuuniteitsgebreksvirus is ook uitgesluit. Die deelnemers was afkomstig van semi-stedelike gebiede in die Noordwes provinsie van Suid-Afrika.

Antropometriese metings van elke deelnemer is na aanleiding van standaard prosedures drievoudig geneem. Sistoliese – en diastoliese bloeddruk en ook harttempo is gemeet in ‘n sittende posisie, na ‘n 10-minute rus tydperk, deur van die OMRON HEM-757 apparaat gebruik te maak. Twee metings is geneem na ‘n 5-minute rus interval. Kardiovaskulêre metings is geneem deur van die Finometer™ apparaat gebruik te maak. Vastende bloedglukose is deur ‘n verpleegkundige direk in die Metaboliese eenheid gemeet deur van ‘n ensiematiese metode gebruik te maak, om te ondersoek vir diabetes mellitus. ‘n Vastende bloedmonster is geneem van die antebragiale vena deur van ‘n

gesteriliseerde infusiestel en spuite gebruik te maak. Standaard metodes is gebruik om plasma en serum monsters voor te berei en is gestoor by -80ºC tot analise. Serum reaktiewe suurstofspesies (RSS) is bepaal met 'n *"high-throughput spectrophotometric assay"* waar een eenheid gelykstaande is aan 1mg/liter H_2O_2. VEGF en Ang-2 is bepaal deur *"enzyme-linked immunosorbant assays (ELISA)".*

Resultate

Wanneer daar gekyk word na die karaktereienskappe van die swart en wit groepe, vertoon die swart mense 'n hoër bloeddrukstatus ($p<0.001$) en ook hoër vlakke van RSS ($p=0.002$), VEGF ($p=0.002$) en Ang-2 ($p<0.001$). Hierdie groep bevat ook meer rokers ($p<0.001$) en hipertensiewe individue ($p<0.001$). Die inneem van anti-hipertensiewe middels was hoër by die wit groep ($P<0.001$).

In die enkel regressie analise was daar geen betekenisvolle korrelasies tussen VEGF of Ang-2 met bloeddruk in die swart en wit groepe nie. RSS het betekenisvol gekorreleer met die twee angiogeniese groeifaktore in beide mans groepe, maar dit was sterker in die swart mans (beide $r=0.33$; $p<0.001$) en swakker in die wit mans ($r=0.16$; $p=0.04$ en $r=0.16$; $p=0.07$). RSS het ook betekenisvol gekorreleer met Ang-2 in die swart vroue ($r=0.26$; $p=0.003$). RSS het swak, maar betekenisvol geassosieer met diastoliese bloeddruk in die wit vroue ($r=0.15$; $p=0.03$).

VEGF en Ang-2 is in kwartiele van RSS gestip en daar is vir ouderdom en liggaamsmassa indeks gekorrigeer. In alle gevalle het die swart mans en vroue betekenisvolle assosiasies getoon van VEGF en Ang-2 met RSS ($p<0.05$), behalwe vir die assosiasie tussen VEGF en RSS in die swart vroue ($p=0.80$). In teenstelling hiermee is geen betekenisvolle assosiasies voorgestel vir die wit groepe nie.

Om die betekenisvolle assosiasies in die swart groepe verder te ondersoek is daar 'n meervoudige regressie analise gedoen met bloeddruk of merkers van angiogenese as afhanklike veranderlikes. Die assosiasies tussen albei angiogeniese groeifaktore met RSS in die swart mans (beide $p=0.014$) en Ang-2 met RSS in die swart vroue ($p=0.025$) is bevestig nadat daar vir sekere veranderlikes, wat die assosiasies kan beïnvloed, gekorrigeer is. Geen assosiasies is gevind tussen die angiogeniese groeifaktore of RSS met bloeddruk nie.

Gevolgtrekking

Ons het in hierdie studie, wat angiogeniese groeifaktore en oksidatiewe stress behels, gevind dat Ang-2 betekenisvol assosieer met oksidatiewe stres slegs in die swart mans en vroue. Addisioneel is VEGF ook met oksidatiewe stress slegs in die swart mans geassosieer. Hierdie assosiasies is in beide wit groepe nie gevind nie. Die sterk assosiasie wat gevind is in die swart populasie, dra moontlik by tot die bestaande hoë risiko vir kardiovaskulêre siektes in hierdie populasie.

Preface

This dissertation consists of four chapters presented in the article format as approved by the North-West University. Chapter one provides an introduction containing a short background and problem statement motivating the purpose of this study and knowledge needed for interpretation of the data. Chapter two contains a complete literature overview of the topic, a detailed summary as well as aims, objectives and hypotheses to clarify the purpose of the study. Chapter three contains the authors' instructions as provided by the Journal of Human Hypertension and an abstract of the article, followed by a complete manuscript to be submitted for publication. The manuscript consists of an introduction, methods, results and discussion. Chapter 4 contains the summary of the main findings and recommendations for future research. After every chapter appropriate references are provided in the format required by the Journal of Human Hypertension.

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List of abbreviations

Ang	Angiopoietin
ADMA	Asymmetric dimethylarginine
Cwk	Windkessel arterial compliance
DBP	Diastolic blood pressure
DMPA	Depot medroxyprogesterone acetate
D-ROM	Derivatives of reactive oxygen metabolites test
HIV	Human immunodeficiency virus
hs-CRP	High-sensitivity C-reactive protein
iNOS	Inducing nitric oxide synthase
LDL	Low density lipoproteins
MAP	Mean arterial pressure
NO	Nitric oxide
NOx	NADPH oxidase
ROS	Reactive oxygen species
SAfrEIC	South African study on the influence of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function
SBP	Systolic blood pressure
SOD	Superoxide dismutase
TPR	Total peripheral resistance
VEGF	Vascular endothelial growth factor

Chapter One

Introduction

Background and Motivation

Compared to their Caucasian counterparts, black South Africans and African Americans have shown increased prevalence of hypertension and stroke.[1,2] Cardiovascular disease is the leading cause of death in the Western world of which hypertension is the leading cause of preventable death and stroke and the second most common cause of death worldwide.[3-5] Common causes for cardiovascular diseases are obesity and diabetes mellitus, which are both increased in black South Africans.[2,6-8] In hypertensive individuals it has been shown that specific factors such as oxidative stress and angiogenic growth factors are elevated.[9-12]

In the vasculature, reactive oxygen species (ROS) are produced by endothelial and smooth muscle cells and are formed as by-products during normal cellular metabolism.[10,13] Physiologically ROS is involved in regulating vascular function, promoting cell growth, migration and differentiation. However, elevated levels of ROS cause tissue injury through cellular dysfunction and destruction.[10,13,14] ROS can also cause an increase in angiogenic growth factors, leading to endothelial cell proliferation and migration and therefore play a fundamental role in angiogenesis.[14] On balance, ROS can also be stimulated by angiogenic growth factors to cause endothelial cell proliferation and migration.[11,14]

Angiogenesis is the formation of new blood vessels and is controlled by pro- and anti-angiogenic growth factors.[15,16] Amongst various others, these growth factors include vascular endothelial growth factor (VEGF) and the angiopoietins.[17] VEGF-A is the most extensively studied of the five isoforms. Of this isoform, at least five splicing forms exist of which $VEGF_{165}$, also referred to as VEGF-A, has been studied most extensively in the cardiovascular system.[9,18,19] This growth factor binds to two transmembrane receptors, VEGFR-1 and VEGFR-2, which are expressed primarily on endothelial cells.[9]

Four known types of angiopoietin exist. Angiopoietin-2 (Ang-2) is mainly produced by vascular endothelial cells and its receptor, Tie-2, is expressed primarily by the same producing cells.[9,11] Where Ang-1 is a protective growth factor, Ang-2 and VEGF cause an increase in vascular permeability and destabilisation of vessel integrity leading to endothelial cell migration and proliferation and finally vessel sprouting.[9,16,20]

When investigating angiogenic growth factors, oxidative stress and cardiovascular disease states, important aspects to consider seem to be gender and ethnicity. Comparing men and women, men show higher levels of blood pressure and oxidative stress, as well as a higher prevalence of cardiovascular disease when compared to pre-menopausal women.[21,22] Angiogenesis may also be influenced by gender-specific influences as it was found that male dogs have higher VEGF levels compared to the females.[23] Dehydrotestosterone causes an increase in angiogenic processes in male endothelial cells.[24] In addition to this, a sexual dimorphism exist in serum levels of Ang-2 as women show higher levels of Ang-2 compared to men due to the modulation of this growth factor and its Tie-2 receptor by estrogen.[8,11,25]

Oxidative stress levels are also higher in African Americans compared to Caucasians,[26] however literature regarding angiogenic growth factors (VEGF and Ang-2) and oxidative stress in black South Africans, as well as literature on the association between angiogenesis and oxidative stress levels in this same population is limited. It is unknown whether these aspects are related to the high prevalence of cardiovascular disease in black South Africans.

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Chapter Two

Literature Study:

Angiogenesis, oxidative stress and cardiovascular disease

1. Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vessels or the formation of new vessels by intravascular subdivision and involves cytokines and pro- and anti-angiogenic growth factors.[1-7] It has a role to play in metastasis, embryonic development, wound healing, tissue remodelling and underlies pathogeneses such as tumour growth and atherosclerosis.[3,4]

Endothelial cells of pre-existing vessels are activated by angiogenic stimuli that causes vasodilatation, increased vascular permeability and the degradation/disruption of the endothelial cell basement membrane.[1,2] This is caused by matrix metalloproteinases and plasminogen.[1] Matrix metalloproteinases are potent regulators of angiogenesis, and plasminogen is a precursor of plasmin, a degrader of fibrinogen.[8,9] This causes cells to proliferate and cytoplasmatic processes to extend from the activated endothelial cells and migrate out of the capillary wall directing the growth/sprouting into the extravascular spaces toward the angiogenic stimuli.[1,2] The endothelial cells secrete platelet-derived growth factor, which recruits mesenchymally derived fibroblasts and smooth muscle cells to cover the endothelial tube. This causes endothelial cell differentiation and creates a thicker, non-leaking vessel.[1] After the proliferation, elongation and alignment of endothelial cells, follows the formation of capillary sprouts that develop a lumen. This tubular structure anastomose with neighbouring vessels which then forms loops that canalise and allow blood-flow.[1,2] The maturation and final stage consists of vessel remodelling by stabilisation, regression and the formation of the basement membrane. It seems as if angiogenesis is an organ-specific process that relies on the stage of the microvascular network.[2]

Vasculogenesis is the organisation of endothelial cells into luminal structures after their differentiation from mesodermal precursors (embryonic mesenchymal cells).[1,2] This process therefore takes place mainly during embryonic development.[2] Arteriogenesis is the rapid proliferation and maturation of pre-existing collateral vessels or may reflect new formation of mature vessels.[2,10] However, a combination of angiogenic growth factors and other growth factors are needed in arteriogenesis for vessel maturation and stabilisation, but this is a poorly understood process.[10,11]

1.1 Angiogenic growth factors

Angiogenesis is an extremely synchronised process. Vascular endothelial growth factor (VEGF) and angiopoietins are secreted and their receptors expressed by various cell types and are the main coordinators for angiogenesis.[2,12] Their role in angiogenesis is initiation, rate establishment and extent of angiogenesis.[2] VEGF and angiopoietin-2 (Ang-2) are involved in pathophysiological conditions, whereas Angiopoietin-1 (Ang-1) is a cell protecting growth factor.[2,5,13] In line with the aim of this study, the authors will mainly focus on VEGF-A and Ang-2, which will subsequently be discussed in more detail.

1.1.1 Vascular endothelial growth factor

The role of VEGF in angiogenesis was demonstrated in VEGF-receptor deficient mice that lacked sufficient blood vessel formation.[14] This was also supported by the abnormal vascular development in embryos that lacked VEGF.[15]

VEGF is a multifunctional, mitogenic peptide that causes receptor-mediated endothelial cell proliferation and migration. It increases cell permeability and has a part to play in maintaining mature vessels, in addition to angiogenesis.[2,5,6,16] This growth factor was shown to be involved under physiological conditions as well as pathophysiologically in processes such as neovascularisation.[2,16] The inhibition of VEGF or its effects causes repression of neovascularisation.[2] After injury, other growth factors, such as fibroblast growth factor, cytokines and other molecules cause angiogenesis either directly or indirectly through stimulation by VEGF, which then apply autocrine or paracrine effects on vascular cells. However, uncontrolled angiogenesis is inhibited by strict control of this peptide.[1,2]

VEGF has a half-life of 10 minutes to 6 hours, binds heparin and has a similar structure to platelet-derived growth factor.[2,17] At least five isoforms of VEGF exist, VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D as well as VEGF-E, of which VEGF-A has been studied most extensively. These are glycoproteins and can bind to three existing VEGF receptors.[2] VEGF binds to two transmembrane receptors, VEGFR-1 and VEGFR-2, which are primarily expressed on endothelial cells.[2,3,5,15,18] These receptors are also found on other cells, including aortic smooth muscle cells. The third receptor, VEGFR-3 binds only VEGF-C and VEGF-D in lymphatic vessels. The effects are mediated

through transmembrane receptor tyrosine kinases.[2] In contrast with this, VEGF is secreted by a variety of cells.[15] VEGF-A has at least five splicing forms, namely: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. In the cardiovascular system, $VEGF_{165}$, often referred to as VEGF-A, has been studied most extensively.[2,10]

1.1.2 Angiopoietins

VEGF and angiopoietins act together to cause proliferation and/or regression of blood vessels as the activities of VEGF and angiopoietins are closely linked and seem to interact with each other.[5,12] Angiopoietin is a growth factor involved in developmental and pathophysiological angiogenesis, as well as vasculogenesis.[2,8,13,19] Four angiopoietins (Ang) exist: Ang-1, Ang-2, Ang-3, Ang-4.[2,13] However, Ang-3 seem to be only present in mice.[2] The receptor for angiopoietins is tyrosine kinase Tie-2 receptor.[2,5,8,13,20] This receptor is vascular endothelium-specific, however a soluble form can be found in human biological fluids. In addition to this, Ang-2 is produced mainly by vascular endothelial cells.[20]

On their own, angiopoietins do not cause endothelial cell proliferation. Ang-1 causes endothelial cell sprouting and survival through the inhibition of endothelial cell apoptosis.[5,6,13,16,19,21] It causes stabilisation of interaction between endothelial cells and supporting perivascular cells and has a prominent role to play in vascular network maturation.[2,6,13,16,19] Ang-1 also regulates integrity, and prevents capillary leakage by restricting the permeability effects of VEGF.[5,6,13,19,22]

On the other hand, Ang-2 serves as an antagonist of Ang-1 by blocking Ang-1 induced phosphorylation of Tie-2 and reducing signalling.[2,5,8,12,19,22] However, depending on the cell type and context, this isoform can also serve as an agonist for the Tie-2 receptor.[5] Ang-1 and Ang-2 may also change cellular survival pathways in the absence of the Tie-2 receptor with the help of integrins.[19] Defective embryonic vascular development have been demonstrated in both Tie-2 or Ang-1 gene deficient mice.[2]

Ang-2 increases vascular permeability and causes destabilisation of vessel integrity, which then leads to vessel sprouting in response to VEGF.[2,5,6,8,13] In combination with VEGF, Ang-2 promotes neovascularisation, assists in endothelial cell migration and proliferation. This can favour endothelial abnormalities, increase capillary diameter and

cause remodelling of the basal lamina.[2,5,6,21,22] In the absence of VEGF, Ang-2 causes endothelial cell death and vascular regression.[5,6,8,19,21,22] Separately, Ang-2 and VEGF do not cause endothelial cell sprouting and Ang-1 together with VEGF only increases perfusion, but not vessel length. Also, Peters et al.[5] found that VEGF and Ang-2 together results in five times the permeability than VEGF alone.[5] It is proposed that Ang-1 may offset VEGF-induced angiogenesis, making it anti-angiogenic.[12] VEGF can bind to the angiopoietin receptor Tie-1, as well as cause an increase in Ang-2 expression in endothelial cells. Tie-1 and Tie-2 receptors are similar as there are heterodimers between them. Therefore, VEGF can have a direct effect on the signalling pathways of angiopoietins.[5] A combination of Ang-1, Ang-2 and VEGF and other anti- and pro-angiogenic factors must be coordinated and act synergistically for successful angiogenesis and to accumulate functional blood vessels.[2,4,17]

2. Oxidative stress

Normal cellular metabolism produces free radicals as by-products. However, an imbalance in the production of reactive oxygen species (ROS) and cellular defence mechanisms causes cellular dysfunction and destruction leading to tissue injury.[23] Under physiological conditions, ROS are involved in cell signalling to regulate vascular function and are produced, among others, by endothelial cells, smooth muscle cells and the adventitial layer of the blood vessel.[24-26] However, ROS can also affect ion channels, ATPases and exchangers, causing alterations in intracellular calcium homeostasis. This leads to temporary calcium overload causing decreased myofilament sensitivity to calcium, excitation-contraction uncoupling and changes in ion movement over the sarcolemma.[27] It can also cause smooth muscle cell hypertrophy and hyperplasia and promote a vascular pro-inflammatory state.[26] At low levels, ROS can cause cell growth, migration, differentiation, gene expression and physiological repair after injury. However, at high levels, ROS are mutagenic and cytotoxic, causing apoptosis and senescence.[3]

Some ROS include superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide (NO) and peroxynitrite ($ONOO^-$).[3,24] Oxygen is a vital substrate and abundant molecule in the biological system. It is a radical with two unpaired electrons that can undergo electron transfer and forms superoxide with the reduction of one electron. This is then either dismutated non-enzymatically to H_2O_2 or enzymatically in a

reaction catalysed by the enzyme superoxide dismutase (SOD).[3,28] Hydroxyl radicals are then formed in further reactions.[3] Hydrogen peroxide can be scavenged by catalase to form water or by glutathione peroxidase in the presence of reduced gluthathione.[26] The potent oxidant, peroxynitrite is formed when superoxide reacts with NO.[3,28,29] NO is protective and the reduction of its availability can cause pathophysiological conditions.[3]

2.1 Difficulties in assessing oxidative stress

Oxidative stress can be seen as an essential marker of general health. Cutler et al.[30] gave a fully detailed summary of the wide array of markers of oxidative stress that is available and can be assessed through blood samples, using serum, plasma, lymphocytes and others or through urine samples, using 24 hour samples, overnight fasting or first morning void, as well as using breath samples taken before and after a controlled exercise at about 70% VO_{2max}. However, they stated that it is difficult to choose an array of markers of oxidative stress, as there are discrepancies in assays regarding interference of the analysis in samples as well as the different levels of accuracy, precision, bio variation and efficiency. Simplicity and costs can also not be left out in making this decision.[30] One such technique that makes use of complicated and costly instruments is the electron spin resonance spectrometer.[31] Another problem in measuring oxidative stress is the sample chosen, as some data obtained in certain samples need to be normalised to correct for differences, and certain samples need either freezing and defrosting or heating of the samples before they can be analysed.[30] It is also often difficult using routine measurements of ROS in clinical laboratories as it is often required to use large numbers of serum samples.[31] For these reasons Hayashi et al.[31] proposed a new method of assessing ROS levels in serum by using a high-throughput spectrophotometric assay that allows them to analyse many serum samples cost-effectively with high reproducibility, consistent accuracy and accuracy while using smaller amounts of sera and reagents. This assay system is based upon modification of the conventional derivatives of reactive oxygen metabolites test (D-ROM). This improved assay requires only 5 minutes or less for measuring 96 samples with 5 µl volume per sample compared to the D-ROM test that requires 9 minutes or more for measuring one sample with 20 µl volume required for a single measurement. Therefore, this new method of measuring ROS levels is simpler, requiring less time, smaller amounts of serum and allows for a large number of samples being measured simultaneously.[31]

3. The interaction of angiogenesis and oxidative stress

ROS and therefore oxidative stress play vital roles in angiogenesis. It stimulates endothelial cell proliferation and migration in response to hypoxia, ischemia, VEGF and Ang-1.[3,4] ROS also cause angiogenesis through the stimulation of VEGF, Ang-1, and Ang-2 which then again cause proliferation, migration and tubular morphogenesis in endothelial cells.[3,20] In addition to this, ROS cause angiogenesis through enzymes such as NADPH oxidase (NOx) that increase ROS and cause activation of redox signalling pathways leading to an angiogenic response in endothelial cells.[3,32] Ushio-Fukai and Nakamura state that growth factors, including VEGF and Ang-1, cytokines, like tumor necrosis factor-α, shear stress, hypoxia, endothelin-1 and agonists like angiotensin II can stimulate NADPH oxidase, thus increasing ROS.[3,24,28] Superoxide dismutase can be either a pro-angiogenic enzyme by generating hydrogen peroxide and thereby increasing VEGF synthesis, or it can be anti-angiogenic as extracellular superoxide dismutase protects against the overproduction of superoxide.[3,4] Increased NO and peroxynitrite radical formation are markers of oxidative stress and they significantly correlate with angiogenic markers, thus supporting the role of oxidative stress in angiogenesis.[4]

Sihvo and colleagues[33] investigated the role of oxidative stress and angiogenesis in Barrett esophagus, a complication during gastroesophageal reflux disease. This is a premalignant condition for esophageal adenocarcinoma. During this disease there is a simultaneous increase in oxidative stress and angiogenesis. They suggested oxidative stress to be a pathway for the onset and process of angiogenesis. They also found an increase in superoxide dismutase (SOD) activity in this disease and this increases the ability to counter oxidative stress. Here, again it is stated that superoxide can induce angiogenesis, but can also increase hydrogen peroxide, increasing oxidative stress. However, different subtypes of superoxide may have different effects on angiogenesis. Inflammation, also seen in this disease, causes an increase in VEGF, fibroblast growth factor, Ang-1 and transforming growth factor, which are all activators of angiogenesis.[33] However, they failed in this study to give an in-depth process or mechanism by which oxidative stress causes angiogenesis.

Contributing to the above, Khatri et al.[32] found that smooth muscle cells exhibiting oxidative stress, predominantly hydrogen peroxide, expressed high levels of VEGF and matrix metalloproteinases, both being angiogenic potentiators. In this study, they

induced experimental lesions, which added to the progressive increase in VEGF and hypoxia inducible factor-1α. This increase in hypoxia inducible factor-1α is a ROS-sensitive, hypoxia-independent mechanism.[32]

Laurent et al.[34] found that the accumulation of ROS in pancreatic β-cells causes an increase in pancreatic angiogenesis. One pathway causing this effect is an increase in hydrogen peroxide, leading to the stabilisation of hypoxia inducible factor-1α in β-islets, causing stimulation of VEGF-A and therefore vascularisation and increased insulin expression.[34]

4. Angiogenesis and oxidative stress in disease states

4.1 Cardiovascular disease

The leading cause of death in the Western world is cardiovascular diseases. This includes hypertension, coronary artery disease, congestive heart failure and stroke.[28,35] Overall, hypertension is the leading cause of preventable death and stroke and is the second most common cause of death. A reduction in hypertension prevalence, through prevention and treatment, reduces morbidity and mortality.[35-37]

4.1.1 Angiogenesis, oxidative stress and hypertension

VEGF is a contributor to the regulation of blood pressure and insufficient regulation of VEGF in hypertension leads to an increase in VEGF in hypertensive patients.[35,38-40] However, it is possible that this increase in VEGF may only reflect endothelial damage caused by hypertension.[41] Zorena et al.[42] found that in patients with type 1 diabetes with hypertension and other complications, VEGF levels were higher compared to those without hypertension as well as to healthy subjects.[42] Interestingly anti-hypertensive medication have shown effects on angiogenesis; however this effect is dependent on the anti-hypertensive medication used, as some medication inhibit angiogenesis and other increase angiogenesis.[35]

Contradictory to the above, the use of VEGF-pathway inhibitors results in hypertension as an adverse and frequent side effect.[39,40,43] This effect seems to be dose-dependent.[40,43] Pathophysiological mechanisms for this association is yet to be fully explained, however a few have been proposed.[39,43] VEGF causes an increase in NO,[2,29] therefore, one possible mechanism is that these VEGF-inhibitors cause a decrease in

NO and an increase in the vasoconstrictor, endothelin-1, resulting in increased vascular resistance and a hypertensive response.[39,40] Another possible mechanism is a decrease in the number of small arteries and arterioles after treatment with VEGF-inhibitors (rarefaction).[40] This feature is an early event in hypertension. Also increased after treatment, is arterial stiffness, contributing to hypertension.[40] These mechanisms are only proposals as there may be other contributing mechanisms underlying the development of hypertension. A full understanding of the association between VEGF-inhibitors and hypertension is still absent.[39,40] Treatment involving VEGF-inhibitors are used in a variety of cancers, therefore, this association with hypertension may be influenced by other underlying diseases.[39,40,43]

An increase in Ang-2 in hypertensive individuals have also been found.[18,20,44] However the mechanisms explaining the increase in VEGF and Ang-2 in hypertension as well the association between angiogenesis and oxidative stress in hypertensive individuals is still lacking. It is also unknown if this increase in growth factors, such as Ang-2 in hypertension is a cause or an effect of hypertension.[37]

Oxidative stress is closely involved in the pathogenesis of hypertension. Reactive oxygen species such as superoxide and hydrogen peroxide are increased and activities of superoxide dismutase and catalase are decreased in cardiovascular disease.[24,26] The increased activity of the NADPH oxidase pathway can also be seen in the pathology of hypertension as there is an increase in angiotensin II in hypertension and thus an increase in NADPH oxidase through the stimulation of the angiotensin I receptor.[24,28] However, the NADPH oxidase pathway should not be accepted as the main source of excess superoxide, as this may not be the case.[26] Peripheral resistance may also increase through an increase in peroxynitrite, thereby decreasing NO and increasing vasoconstriction.[24] A wide variety of mechanisms exist by which oxidative stress may lead to hypertension. There is however discrepancies whether oxidative stress is a cause or a result of hypertension, however Grossman supports the latter.[45]

Despite the previous mentioned association, Keaney et al.[46] did not find an association between creatinine-indexed urinary 8-epi-$PGF_{2\alpha}$ (a marker of oxidative stress) and hypertension. However, one third of their subjects were receiving anti-hypertensive medication. After adjusting for medication usage, they still did not observe an

association between blood pressure and this marker of oxidative stress. When comparing their data to previous animal studies, they suggested that the association between hypertension and oxidative stress might only apply in certain hypertension states.[46]

4.1.2 Angiogenesis and oxidative stress in the vasculature

Although VEGF is secreted by a variety of cells, two transmembrane receptors, VEGFR-1 and VEGFR-2, which binds VEGF, are primarily expressed on endothelial cells.[2,3,5,15,18] In addition to this, VEGF is a regulator of endothelial and vascular function.[39] Ang-2 is produced mainly by vascular endothelial cells and its receptor, the Tie-2 receptor, is vascular endothelium-specific.[20]

The release of NO is increased by VEGF, by inducing NO synthase (iNOS) production during angiogenesis.[2,29] NO has a fundamental role to play here as can be seen in NOS deficient mice, that lack adequate angiogenesis. Despite this, NO has a negative feedback regulatory effect on VEGF through a NO-induced decreased binding of transcription activator protein-1 by protein kinase C. This results in a decreased stimulation of the promoter region on the VEGF gene.[2] However, Hamed et al.[4] stated that generally an increase in NO inhibit angiogenesis (due to inflammatory responses), whereas low NO in response to angiogenic growth factors, such as Ang-2, stimulate angiogenesis, causing pathology.[4,47] Therefore, increased VEGF can be seen in atherosclerotic and hypertensive patients, in which NO is decreased.[2,22]

Oxidative stress is also related to endothelial dysfunction through an increase in superoxide, activation of NADPH oxidase and uncoupling of iNOS[24,25] and is therefore resultantly associated to other cardiovascular diseases.[24,25,28] Through its association with endothelial dysfunction, oxidative stress may also be associated with cardiovascular events.[25] Oxidative stress results in atherosclerosis through oxidised low density lipoproteins and the uncoupling of iNOS, NADPH oxidase-, xanthine oxidase-, lipoxygenases-, and mitochondrial pathways. These pathways are also the main source of ROS in the vasculature.[24-26,28,48] A reduction in NO can also be seen in endothelial dysfunction in atherosclerosis as superoxide is increased in this disease.[24,25] Interestingly, not only does iNOS produce NO, but also superoxide.[25,26]

Inflammation is also actively involved in adult angiogenesis. Some mediators contributing to angiogenesis are insulin-like growth factor-1, tumor necrosis factor-α, interleukin-1α and substance P.[10] Contributing to the uncertainty regarding cause-and-effect, angiopoietins have a role to play in the regulation of inflammation. Ang-1 has anti-inflammatory properties by inhibiting the transcription factor (NF)-κB (nuclear factor) and decreasing leukocyte adhesion molecules.[13,22] Ang-2 promotes inflammation by making the endothelium sensitive to tumor necrosis factor-α, which is a pro-inflammatory cytokine.[13]

Both angiogenic growth factors, VEGF and Ang-2, as well as ROS are associated with vascular remodelling.[44,49-52] Vascular remodelling can be defined as the vessel wall undergoing structural and functional changes and leads to cardiovascular disease.[44]

4.1.3 Angiogenesis and oxidative stress in the heart

VEGF is involved in cardiovascular development through stem cell differentiation into cardiomyocytes, as well as stem cell migration and development. However, VEGF also seems to play a role in cardiovascular diseases as it again enters the adult cardiomyocyte, causing heart cell division and promoting cardiac hypertrophy.[53] VEGF levels are also increased in hypertension, atherosclerosis, patients with congestive heart failure and acute coronary disease, together with Ang-2 in the latter two conditions.[2,13,21,22] This is proven by the expression of both VEGF and Ang-2 in the coordinated event of coronary angiogenesis.[12]

Angiogenesis is increased in the subacute, acute and chronic phases after acute myocardial infarction. An increase in VEGF levels is seen in patients suffering from myocardial infarction and ischemia and is therefore an independent predictor of nonfatal and fatal myocardial infarction and death.[12] Also increased in patients with coronary disease and in those after acute myocardial infarction, are the levels of circulating Tie-2 receptors. However, according to Lee et al.[12] the relationship between VEGF, Ang-1, Ang-2 and the Tie-2 receptor in these patients are unknown. They confirmed in their study increased levels of VEGF, Ang-2 and Tie-2 receptor, but found no increase in Ang-1 in patients with acute coronary syndrome and myocardial damage.[12]

Oxidative stress is a possible cause for heart failure. This is caused through the increase of NADPH oxidase by angiotensin II and also through an increase in NADPH by tumor necrosis factor-α.[24-26,28] Other oxidative pathways through which heart failure is caused are: the mitochondria, xanthine oxidase, an increase in superoxide, a reduction in NO bioavailability and an increase in peroxynitrite.[25,28]

Oxygen is an important regulator of VEGF. Hypoxia is a condition seen in various pathophysiological conditions, including atherosclerosis, which stimulate angiogenesis through an increased expression of VEGF, in turn causing neovascularisation.[2,21] During hypoxia, a protein is induced called hypoxia-inducible factor. This protein causes up-regulation of VEGF mRNA by binding to a hypoxia-inducible factor-1 binding-site in the VEGF promoter region. This protein also causes up-regulation of VEGFR-1.[2,10] Felmeden et al.[2] states that the increase in VEGF may also be due to features seen in hypoxia. These include tissue damage, necrosis and apoptosis that cause events leading to increase in VEGF. VEGF levels return to baseline within twenty-four hours after normal oxygen levels have been established. In contradiction with this, hyperoxia causes a decrease in VEGF.[2]

Heart failure is a cause for tissue ischemia, which is a stimulus for angiogenesis.[21] As stated above, hypoxia causes an increase in hypoxia-inducible factor, which causes an increase in VEGF and its receptors.[2,10] This also occurs in ischemic regions of the heart, causing an increase in capillary density.[10] This is proven by the rapid increase in VEGF mRNA, protein and its receptors within minutes in the myocardium following ischemia/hypoxia.[12] Lim et al.[22] speculated that cardiovascular outcome might be improved by lowering angiogenic growth factors.[22]

On the other hand, growth factors such as VEGF can also be used in a therapeutical manner.[11] Growth factors are used in heart and peripheral vascular diseases to stimulate vessel growth where blood flow is insufficient causing ischemia. Treatment with different isoforms of VEGF in the myocardium of patients showed improvement in ischemia and also reduced angina.[11]

Despite the possible benefit of angiogenic therapy, vessels stimulated to form in this way are more likely to regress, as the use of VEGF-A alone makes newly formed vessels

unstable and leaky, unless they are modulated into mature stable vessels by arteriogenesis.[11] Also, a side effect dependant on dosage of VEGF and fibroblast growth factor-2, is systemic hypotension. However, hypotension caused by fibroblast growth factor-2 is higher than that caused by VEGF.[10]

4.1.4 Angiogenesis and oxidative stress in the brain

After stroke, ischemia occurs in the brain, which leads to cerebral blood vessel damage that can cause cerebral edema and hemorrhagic transformation. Tissue damage can increase if reperfusion occurs in the blood vessels.[29] Oxidative stress has been seen after brain ischemia and reperfusion and oxygen radicals such as hydrogen peroxide, hydroxyl radical and superoxide increase during this reperfusion.[28,29] The superoxide causes the vascular response to CO_2 and vasodilators to change; it increases platelet aggregability and increases the permeability of the endothelium as well as the blood brain barrier.[29] As mentioned, superoxide and NO react to form peroxynitrite,[3,29] which further causes damage by inflammation and apoptosis.[29] However, an overexpression of superoxide dismutase in mice show neuronal protection.[28]

After stroke, pro-angiogenic factors promote neurogenesis and help with the inadequate perfusion of the collateral vasculature. Zhu et al.[8] found in their study that Ang-2 on its own did not promote microvessel increase but together with VEGF, an increase in microvessel counts could be seen. Therefore these two peptides work synergistically to cause brain angiogenesis. However, Ang-2 may also have a disruptive role in the integrity of the blood brain barrier through an increase in blood brain barrier permeability.[8] Ang-1 on the other hand, has a protective role in blood brain barrier permeability, but decrease after ischemia, thus contributing to the increased permeability. However, in the subacute and chronic phase of stroke, VEGF may have a protective role, but as stated by Fagan et al., should be further investigated.[29]

4.2 Obesity

Angiogenesis and the development of adipose tissue (adipogenesis) are functionally linked. Angiogenesis is enhanced in obesity, as it is necessary for the expansion of adipose tissue and its capillary bed for the development of obesity. Inhibiting angiogenesis decreases adipose tissue development.[17,54,55] The inability of adipose

tissue to expand causes an ectopic accumulation of lipids in the liver and skeletal muscle as well as insulin resistance and metabolic disease.[55]

Again contributing to the uncertainty of cause-and-effect, adipocyte differentiation increase angiogenesis and angiogenic growth factors such as VEGF modulate adipocyte differentiation. There is also cross-talk between adipocytes and endothelial cells for angiogenesis and adipocyte differentiation, which are both abundant in adipose tissue.[17,54]

Adipose tissue can stimulate angiogenesis as growth factors such as VEGF, Ang-2 and fibroblast growth factor are increased in human and animal obesity.[54,55] Adipose tissue also seems to express anti-angiogenic factors such as angiostatin and endostatin. Silha et al.[17] confirmed an increase in VEGF (VEGF-C and VEGF-D) and Ang-2 in obese or overweight individuals, but they also found an increase in VEGFR-2 and endostatin. They showed a correlation between VEGF-C and VEGF-D and body mass index.[17]

Adipose tissue have also shown increased expression of NADPH oxidase and therefore increased production of ROS, as well as decreased antioxidant enzymes, including superoxide dismutase, catalase and glutathione peroxidase.[46,56] This suggests that an obese individual is unable to provide appropriate levels of antioxidants to compensate for the production of free radicals.[56] A study done by Keaney et al.[46] show the relation between obesity and systemic oxidative stress. They used creatinine-indexed urinary 8-epi-prostaglandin $F_{2\alpha}$ as an estimation of oxidative stress and found it to be positively associated with indices of obesity, such as body mass index.[46] Njajou et al.[57] used oxidized low density lipoproteins as a marker of oxidative stress and also found it to be associated with obesity in both African Americans and Caucasian groups.[57]

4.3 Diabetes mellitus

Not only hyperglycaemia but also hypoglycaemia causes an increase in plasma VEGF.[2,58,59] This occurs because of an increase in calcium caused by low glucose levels, which in turn causes the activation of protein kinase C and activation of transcription activator protein-1 VEGF expression. The hyperglycaemic state also results in increased VEGF through a protein kinase C mechanism.[2] Thus, as Felmeden et al.[2] state, further investigation is needed to clarify the association between VEGF,

angiogenesis and diabetes, as it seems that any non-euglycaemia state causes an increase in VEGF.[2]

Lim et al.[6,22] investigated the change in VEGF, Ang-1 and Ang-2 in diabetes and found that VEGF and Ang-2 were increased in patients with type 2 diabetes, but not Ang-1.[6,22] Insulin also have angiogenic properties partly through the stimulation of VEGF, especially in adipose tissue.[54] In addition to this, angiogenesis also plays a role in modulating insulin production. Pancreatic β-cells also secrete VEGF-A, thereby promoting local vascular development.[34]

Increased oxidative stress can also be seen in both types of diabetes[23-25,28] where it participates in the mechanism for the development and progression of diabetes and diabetic complications. Increased free radicals and a decrease in activity of antioxidants enzymes such as catalase, superoxide dismutase and glutathione peroxidase, contribute to the increased oxidative stress and increase in complication development in diabetics.[23,60] Interesting to note is that β-islets are along with those tissues with the lowest levels of inherent antioxidant defences.[23,34,61]

Hyperglycaemia can lead to oxidative stress in diabetes through the excessive stimulation of the mitochondrial electron transport chain, causing an overproduction of superoxide anions.[48] It also causes the production of superoxide through the activation of NADPH oxidase in vascular cells.[46] Hyperglycaemia also results in inactivation of superoxide dismutase, causing a decrease in scavenging of excessive superoxide.[48] This results in a negative association between glucose concentration and superoxide dismutase activity.[56] Free radicals are also formed when hyperglycaemia causes lipid peroxidation of low density lipoproteins (LDL) by a superoxide-dependant pathway.[46,60] Glucose uses ROS as signalling molecules in glucose-dependant stimulated insulin secretion.[34] Keeping in mind the association between oxidative stress and angiogenesis, the increase in free radical production and decrease in superoxide dismutase during hyperglycaemia results in an increase in VEGF.[4]

Hyperinsulinemia can also lead to oxidative stress by increasing the production of hydrogen peroxide when insulin binds to its receptors. This is also seen in adipose tissue.[46,48,61] Insulin resistance causes a decrease in superoxide dismutase activity,

leading to an increase in superoxide anion concentrations.[56] Hyperinsulinemia also cause stimulation of NADPH oxidase through angiotensin II and increase oxidative stress.[48] Insulin resistance and oxidative stress are linked through the impairment of internalisation of insulin by endothelial cells through oxidative stress. This limits the delivery of insulin to target tissue and hinder GLUT-4-mediated glucose transport.[46,48] Hydrogen peroxide is one such marker of oxidative stress that causes these two features in insulin resistance. Thus, oxidative stress is also a mechanism to cause hyperinsulinemia through insulin resistance.[46] However; oxidative stress is lower in type 2 diabetes mellitus compared to type 1 diabetes mellitus due to metabolic differences between type 1 and type 2.[23]

4.4 Cancer

Angiogenic growth factors, such as VEGF and Ang-2 show increased levels in some types of cancers, as the tumours can produce hypoxia and thus lead to pathophysiological neovascularization.[2,3,5,17,21] VEGF inhibitors can be clinically used for the treatment of cancers.[54] In addition to this, free radicals also have a role to play in cancer as oxidative damage have been seen in the pathogenesis of this disease.[24,34,46] Smoking, a cause for cancer, have shown to increase oxidative stress.[26,62] Compared to non-smokers, the antioxidant activities of glutathione peroxidase, superoxide dismutase and catalase are lower in smokers, as well as passive smokers.[46,63]

5. The context of cardiovascular disease, angiogenesis and oxidative stress in the black South African population

Due to the burden of parasitic diseases, plagued infections and nutritional deficiencies contributing to maternal and perinatal morbidity and mortality being high priority in sub-Saharan African, the prevention of cardiovascular diseases is of far less importance in this region. Hypertension is therefore often underdiagnosed or treatment is too expensive, leaving patients undertreated or untreated.[64]

5.1 General context of cardiovascular disease in South Africa

African Americans and black South Africans show an increased risk for cardiovascular diseases, in particular hypertension and stroke.[65-69] In both the developed and developing world, blood pressure is considered the biggest contributor to death rates.[70] However, when compared to sub-Saharan Africa, African Americans show a higher

prevalence of hypertension.[67] This is because African Americans have been acculturated for hundreds of years, whereas urban Africans have been acculturated only since the turn of the 20th century.[67] It is this acculturing or westernisation/urbanisation that partially accounts for the high prevalence of hypertension and stroke in Africans.[69,71]

Urbanisation is rapidly increasing in South Africa and Africans are being more exposed to a Western diet, therefore discarding their traditional diets. The Western diet, characterised by an increase in saturated fats, is associated with a high prevalence of degenerative diseases.[72] Individuals employed in cities are often exposed to long commuting distances and therefore choose to eat easy-to-prepare or take away foods that are high in fat content. Those who are under-employed may have more time to prepare more traditional foods.[72] In addition to this, low educated women often have lower body mass indexes when compared to higher educated women, possibly because the former are exposed to more manual labour.[64,72]

It is a well-known fact that obesity is associated with hypertension.[67,69] It has previously been found that inactivity, no matter the amount of urbanisation, is associated with increasing obesity and low-intensity exercise twice a week can possibly lower systolic blood pressure significantly in the African population.[72] In addition to this, most obese and overweight African patients perceive themselves as being normal or even underweight. This incorrect perception is associated with the level of education, where those with less education have the most incorrect perception.[68] Overall, apart from hypertension, Africans also show a higher prevalence of obesity and diabetes, particularly among urban African women, also due to urbanisation and westernisation.[65,66,73-78]

When exploring the high prevalence of hypertension in Africans, it is apparent that hypertension is not only a problem of the wealthy, sedentary and obese urban population; even though this has been the accepted norm.[79] It has been shown that Africans with a lower socio-economic status have a higher prevalence of hypertension.[67] This low status is characterised by those who are married, have poor living conditions with large families and less involvement in community life. In addition to this, middle-aged patients with hypertension often limit caring for their own health due to illness and death in their families.[67-69] It is important to note that Africans are the most impoverished

population in South Africa,[72] therefore these factors are not uncommon. However, even though urbanised Africans have higher educational status and better living conditions in formal housing, their diets (as explained above) are significantly more atherogenic. Therefore, improving their socio-economic status may not necessarily lead to an improved nutritional status.[72] Other behavioural factors associated with the high prevalence of hypertension in Africans are alcohol and drug abuse, smoking, lack of sleep, as well as unemployment. There also seems to be a lack of awareness regarding hypertension, the benefits of treatment and also the complexity of treatment and side effects that contribute to the prevalence of hypertension. In addition to this, psychological stress such as anxiety and physiological factors such as increased autonomic nervous system activity may also contribute.[64,67-69]

In addition to this, the cost of care and medication and lack of health insurance or a healthcare provider adds to the hypertension epidemic.[68,69] Healthcare system inadequacies have worrying implications for hypertension. Dennison et al.[68] reported the following: the majority of public sector patients stated that they were not told their blood pressure readings at their previous visit. Despite low blood pressure control rates, less than one change in medication prescriptions was previously found in patient records and 16% of patients were receiving an inadequate supply of medication.[68] In addition to this, a large number of patients do not take their medication on the morning of their hypertension care visit to the community health centre due to long waiting queues. Those on diuretics do not want to lose their place in the queues to go to the bathroom. These problems mentioned are common in the public sector of the healthcare system and less common in the private sector; however those in the latter are more obese and have more diabetes than patients in the public sector.[68]

5.2 Possible ethnic differences in the cause of hypertension

Possible explanations for the increased prevalence of hypertension in the African population have been raised. Some specific explanations include the following:

Melikian et al.[65] found that asymmetric dimethylarginine (ADMA) levels, were higher in African men.[65] ADMA is a competing factor to L-arginine for NO substrate and results in a decrease in NO bioavailability.[80] L-arginine is a precursor of NO and results in an increase in NO levels.[81] Our research group previously found no significant difference in

ADMA levels between African and Caucasian men,[82] however, L-arginine levels were significantly lower in African men.[83] These findings could therefore suggest a decreased bioavailability of NO in Africans, resulting in decreased endothelial function, such as decreased NO-induced vasodilatation, compared to Caucasians.[65,84,85]

Differences in renin levels have also been found between urban and rural populations in South Africa, where the former show low renin levels.[69] Overall, renin is lower in black people and does not increase in response to sodium and volume depletion.[67] Sodium levels in blood cells are also higher and potassium levels lower in black hypertensives, which is associated with a rise in blood pressure. This is due to depression in the sodium pump, additionally leading to low magnesium levels in cells.[69] Sodium loads are excreted more slowly by black subjects.[67] Additionally, a mutation of the sodium channel was found in a subgroup of black South Africans.[69] There also does not seem to be a correlation between plasma renin and aldosterone in urban black South Africans causing a low-renin, low-aldosterone hypertension.[67,69]

In addition to this, ethnicity seems to be a risk factor for oxidative stress as can be seen in African Americans, who show increased oxidative stress compared to Caucasians.[84] This was seen by an increase in hydrogen peroxide and NADPH-derived oxidative stress in this ethnic group. African Americans also show higher plasma superoxide dismutase and overall antioxidant activity compared to Caucasians, as increased oxidative stress causes an increase in antioxidant activity.[84] Literature regarding the physiological mechanisms behind these ethnic differences in oxidative stress are limited.

Very limited information exist on ethnic disparities regarding angiogenesis, particularly in the black population; however, perhaps the association between oxidative stress and angiogenesis should be emphasised again, as oxidative stress leads to an increase in angiogenic growth factors (VEGF and Ang-2) and also, angiogenic growth factors lead to an increase in ROS.[3]

6. Gender differences in cardiovascular disease, angiogenesis and oxidative stress

VEGF and Ang-2 levels and therefore angiogenesis may be influenced by gender.[20,86] Silha et al.[17] reported sexual dimorphism in the serum levels of VEGF-C and VEGF-D,

as well as Ang-2.[17,44] Consistent with this, Kemp et al.[87] found that in male dogs, VEGF levels were higher than in the female dogs. However, women have shown higher plasma levels of Ang-2, but lower levels of soluble Tie-2 receptor. Estrogen/Estradiol and other sex steroids have been shown to play a role in angiogenesis by influencing growth factors such as VEGF and Ang-2.[20,44,87,88] Sieveking et al.[89] found that dehydrotestosterone caused a parallel increase in angiogenic processes in male endothelial cells. This is partially caused by sex differences in androgen receptor expression in the vasculature, as females have a two- to fourfold lower expression of these receptors. Endothelial exposure to dehydrotestosterone in women had no effect on angiogenic properties. One mechanism by which dehydrotestosterone induces angiogenesis in males is through the increase in VEGF upon exposure of endothelial cells to this androgen.[89]

Parallel with higher blood pressure in men, they show higher oxidative stress than premenopausal women, who also show lower levels of cardiovascular diseases.[90-92] Sartori-Valinotti et al.[92] found that apart from the level of oxidative stress in tissue, male spontaneous hypertensive rats' blood pressure is more modulated by oxidative stress than the females' blood pressure. However, a limitation to their study was that they did not interfere with ROS in the female rats by for example lowering its levels and observing whether it would reduce blood pressure in these rats. It is also possible that female spontaneous hypertensive rats have higher tissue levels of superoxide dismutase.[92] Ide and co-workers[93] also found that in young men, oxidative stress was higher compared to age matched, pre-menopausal women.[93]

After menopause, oxidative stress levels and hypertension in women are greater than in men. In parallel with this, the risk for cardiovascular events also increases in women after menopause.[90,91] Even though oxidative stress is increased in coronary artery disease, Vassalle et al. found that oxidative stress was even more increased in postmenopausal women with coronary artery disease.[90]

Consistent with the above, postmenopausal women show increased blood pressure and oxidative stress compared to premenopausal women.[91] Estrogen therefore has a protective role in cardiovascular disease, but is decreased after menopause.[94] Estrogens have antioxidant properties as this hormone can reduce expression of NADPH oxidase

and lower the levels of superoxide, increase the expression of superoxide dismutase and glutathione peroxidase and inhibit lipid oxidation.[91,95,96] The antioxidant effects of estrogen are countered by progestins through the activation of NADPH oxidase and the inhibition of superoxide dismutase. Pincemail et al.[95] stated this effect of estrogen is not supported by all studies and that differences in findings might be due to chemical heterogeneity in the estrogen family, concentration differences and differences in the environment in which they are found.[95,96]

To contribute, again using creatinine-indexed urinary 8-epi-prostaglandin $F_{2\alpha}$ as a marker of oxidative stress, Keaney et al. found this marker to be higher in women than in men at similar levels of urinary creatinine, aged between 30 and 90 years.[46] Some of these women were not in their menopause phase, therefore a possible reason for the overall higher oxidative stress levels in the women might be the use of contraceptive medication as it seems that contraceptive medication may increase ROS in women.[97] However, this is highly speculative as it was not specified in this study if contraceptive medication was used.

6.1 The use of oral contraception and its effect on oxidative stress

Discrepancies exist about the effect of oral contraceptive medication on oxidative stress. Some studies found blood lipid peroxides to be increased and antioxidants (β-carotene) decreased, whereas other studies found increased activity of antioxidant enzymes or no effects on oxidative stress.[95,96] Pincemail and co-workers[95] found in their study that the concentration of β-carotenes was significantly decreased in oral contraceptive users when compared to non-contraceptive users and those using intrauterine devices. In addition to this, they also found a significant increase in lipid peroxides.[95] This finding is supported by De Groote et al.[96] In addition, they found a significant increase in oxidised low-density lipoproteins. They proposed a mechanism involving an increase in copper due to estrogen and proposed the combination of activated oxidative stress pathways with a copper release to initiate a chain reaction leading to the production of superoxide, copper reduction, formation of hydroxyl radicals and lipid peroxidation.[96] Plasma copper, which has pro-oxidant properties, has been shown to be increased in oral contraceptive users. In contrast to the above, Olatunji et al.[98] found oral contraceptives not to have any effect on lipid peroxidation. This might be dependent on the dose and duration used in their study.[98] However, it should be noted that their study was performed on rats.

7. The role of age regarding angiogenesis and oxidative stress

Increasing age results in impairment of angiogenesis due to a decrease in angiogenic growth factors and an increase in anti-angiogenic factors. One important reason for this might be the age-related decrease in eNOS.[4,99] Lieb et al.[44] found that Ang-2 increased and soluble Tie-2 receptor decreased with age, where VEGF on the other hand was found to be decreased.[44,99] Aging also seems to be associated with oxidative stress, as generation of ROS causes degenerative diseases associated with aging.[34,90] However, this is controversial, possibly due to the choice of marker for oxidative stress.[92] Using creatinine-indexed urinary 8-epi-prostaglandin $F_{2\alpha}$ as a marker, Keaney et al.[46] found a negative correlation between age and oxidative stress. They stated that there is an association between age and changes in total body fat mass, circulating free fatty acids and renal function, all of which could alter the value of creatinine-indexed urinary 8-epi-prostaglandin $F_{2\alpha}$.[46]

8. Summary

Angiogenesis is a physiological necessity, but can also be involved in pathophysiological processes and is associated with oxidative stress. An increase in oxidative stress can increase angiogenesis and vice versa. Both oxidative stress and angiogenesis are involved in cardiovascular diseases, including hypertension. Blood pressure, oxidative stress and angiogenesis are influenced by gender as both blood pressure and oxidative stress are higher in men compared to pre-menopausal women. This feature may be influenced by the use of contraceptive medication, resulting in higher oxidative levels in women. However, after menopause, women show higher levels of hypertension and oxidative stress compared to men. VEGF seems to be higher in male subjects, but Ang-2 shows higher plasma levels in women. African Americans and sub-Saharan Africans show a higher prevalence of hypertension when compared to Caucasians. In addition to this, African Americans also show higher levels of oxidative stress. However, literature on oxidative stress levels and the levels of angiogenic growth factor (VEGF and Ang-2) and their relationship with each other and with cardiovascular measurements in black South Africans are limited.

Aims and objectives

To contribute to our understanding of the high prevalence of hypertension in black South Africans, we aimed to determine whether relationships exist between angiogenic growth factors (VEGF and Ang-2) and oxidative stress and secondly to determine their relationship with cardiovascular measurements when comparing African and Caucasian populations.

The objectives were:

1. To compare cardiovascular measurements, reactive oxygen species and angiogenic growth factors between Africans and Caucasians;
2. To investigate the association of oxidative stress with angiogenic growth factors in Africans compared to Caucasians; and
3. To investigate the association of cardiovascular measurements with oxidative stress and angiogenic growth factors in Africans and Caucasians.

Hypotheses

We hypothesised that:

1. Cardiovascular measurements, as well as reactive oxygen species and angiogenic growth factors are elevated in Africans when compared to Caucasians.
2. Oxidative stress show stronger associations with angiogenic growth factors in Africans compared to Caucasians.
3. Cardiovascular measurements are adversely associated with oxidative stress and angiogenic growth factors in Africans and Caucasians, but these associations are stronger in Africans.

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Chapter Three

Oxidative stress and angiogenesis in Africans and Caucasians: The SAfrEIC study

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Journal of Human Hypertension: Authors' instructions

Scope

The editors will consider for publication all suitable papers dealing directly or indirectly with clinical aspects of hypertension, including epidemiology. The journal aims to perform the dual role of increasing knowledge in the field of high blood pressure as well as improving the standard of care of patients.

Original Article

Original articles and research letters must include an extra table to be named 'Summary table', with two parts: firstly, the heading 'What is known about topic', and then secondly: 'What this study adds'. This should be two or three bullet points for each, with one or two short sentence for each bullet point. The objective of this is to provide the reader with a brief, quick and focused summary of your work in the perspective of other data.

Abstract and Keywords

The abstract should not exceed 200 words and three to six keywords should be included to aid web searches after publication.

Introduction

The introduction should assume that the reader is knowledgeable in the field and should therefore be as brief as possible but can include a short historical review where desirable.

Methods

This section should contain sufficient detail, so that all experimental procedures can be reproduced, and include references. Methods, however, that have been published in detail elsewhere should not be described in detail. Authors should provide the name of the manufacturer and their location for any specifically named medical equipment and instruments.

Results

The results section should briefly present the experimental data in text, tables or figures. Tables and figures should not be described extensively in the text.

Discussion

The discussion should focus on the interpretation and the significance of the findings with concise objective comments that describe their relation to other work in the area. It should not repeat information in the results. The final paragraph should highlight the main conclusion(s), and provide some indication of the direction future research should take.

Acknowledgements

This should be brief, and should include sources of support including sponsorship (e.g. university, charity, commercial organization) and sources of material (e.g. novel drugs) not available commercially.

References

Only papers directly related to the article should be cited. Exhaustive lists should be avoided. References should follow the Vancouver format. In the text they should appear as numbers starting at one and at the end of the paper they should be listed (double-spaced) in numerical order corresponding to the order of citation in the text. All authors should be quoted for papers with up to six authors; for papers with more than six authors, the first six only should be quoted, followed by *et al*. Abbreviations for titles of medical periodicals should conform to those used in the latest edition of *Index Medicus*. The first and last page numbers for each reference should be provided. Abstracts and letters must be identified as such. Papers in press and papers already submitted for publication may be included in the list of references but no citation is required for work that is not yet submitted for publication.

For example:

Journal article, up to six authors:

Gasowski J, Fagard RH, Staessen JA, Grodzicki T, Pocock S, Boutitie F et al. Pulsatile blood pressure component as predictor of mortality in hypertension: a meta-analysis of clinical trial control groups. *J Hypertens* 2002; **20**: 145–151.

Chapter in book:

Stanley JC. Renal artery aneurysms. In: Greenfield LJ (ed). *Surgery: Scientific Principles and Practice*. Lippincott Williams & Wilkins: Philadelphia, PA, 2003, pp 1729–1735.

Abstract:

Ochocka AM, Pawelczyk T. Expression of ARHGAP6 in human hypertension. *Eur J Biochem*2003; **1**(Suppl 1): (abstract P3.2–67).

Abstract

Oxidative stress and angiogenesis are related and both have been associated with the development of hypertension. The prevalence of hypertension is most pronounced in black South Africans; however information on the levels of oxidative stress and markers of angiogenesis, and their relationship are unknown in this population. The aim of the study was to explore the possible relationship between markers of angiogenic growth and oxidative stress, as well as their relationship with cardiovascular measurements in Africans. We measured systolic and diastolic blood pressure on the left arm in the sitting position after 10-minutes rest. Human vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2) were measured in serum and reactive oxygen species (ROS) in plasma using a high-throughput spectrophotometric assay. The Africans had higher blood pressure ($p<0.001$), ROS ($p=0.002$), VEGF ($p=0.002$) and Ang-2 ($p<0.001$). ROS correlated significantly with both angiogenic growth factors in the African men (both $r=0.33$; $p<0.001$) and with Ang-2 in the African women ($r=0.26$; $p=0.003$) before and after adjustments. In conclusion, we found Ang-2 to be significantly associated with oxidative stress only in the Africans and VEGF to be linked to oxidative stress only in the African men. This finding possibly adds to the existing high risk of cardiovascular disease in the African population.

Keywords: angiogenesis, oxidative stress, blood pressure, ethnicity.

Introduction

The role of reactive oxygen species (ROS) in the development of cardiovascular diseases has been highlighted numerous times.[1-5] ROS are normal by-products of cellular metabolism.[6] Among others, it is produced by endothelial cells and vascular smooth muscle cells and are physiologically involved in regulating vascular function.[2] However, excessive production of free radicals, such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide (NO) and peroxynitrite ($ONOO^-$), causes injury to the vascular wall through cellular dysfunction, destruction and inflammation.[2,4,7,6]

ROS also play a fundamental role in angiogenesis.[7] Angiogenic growth factors cause an increase in ROS, thus stimulating endothelial cell proliferation and migration. Interestingly, ROS also causes endothelial cell proliferation and migration through the stimulation of angiogenic growth factors, leading to angiogenesis.[7,8]

Angiogenesis is the formation of new blood vessels either sprouting from pre-existing vessels or by intravascular subdivision.[7,9-11] It is an extremely synchronised process involving cytokines, as well as pro- and anti-angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and angiopoietin (Ang).[10,12-15] Both these groups of growth factors are involved in normal physiological and pathophysiological conditions.[10,16,17] VEGF-A (VEGF) is by far the most studied angiogenic growth factor of which the $VEGF_{165}$ splicing form has been studied most extensively in the cardiovascular system.[10,18,19] VEGF binds to receptors that are expressed primarily on endothelial cells.[10] Four angiopoietins (Ang-1 - 4) exist and bind the receptor Tie-2, which is primarily expressed by the vascular endothelium and Ang-2 is produced mainly by vascular endothelial cells.[8,10,17]

The function of VEGF-A and Ang-2 are closely associated with endothelial cell migration and proliferation, as well as an increased vascular permeability, leading to sprouting of new vessels.[12,13,17,20] Increased levels of VEGF, Ang-2 and oxidative stress are evident in cardiovascular diseases[1-3,10,17,21-23] and both VEGF and Ang-2, as well as oxidative stress are closely associated with the development of hypertension.[4,10,19,24]

Cardiovascular diseases are the leading cause of death in the Western world of which hypertension is the leading cause of preventable death and stroke.[1,25,26] Black South Africans show an increased prevalence of hypertension, with a resultant high stroke rate.[27,28] Pertaining to the focus of this paper, data on oxidative stress in black South Africans is extremely limited. However, studies in African Americans have shown increased levels of oxidative stress compared to Caucasians.[29] In addition; there is a dearth of literature on angiogenic growth factors in black South Africans or African Americans. Also lacking is evidence on the association between angiogenesis and oxidative stress in hypertensive individuals.

In order to contribute to our understanding of the high prevalence of hypertension in black South Africans, we aimed to determine whether relationships exist between angiogenic growth factors (VEGF and Ang-2) and oxidative stress and secondly to determine their relationships with cardiovascular measurements in black South Africans and Caucasians.

Methods

This study was a sub-study of the cross-sectional SAfrEIC study (South African study on the influence of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function). Originally the study included 750 African and Caucasian men and women aged 20 to 70 years, from semi-urban areas in the North West Province of South Africa. Pregnant or lactating women and those infected with the human immunodeficiency virus were excluded from this sub-study, resulting in a total of 626 participants.

After all the procedures were explained to the participants, they signed an informed consent form and, if needed, an interpreter was available to translate all the information to the participants in their home language. This study was approved by the Ethics committee of the North-West University (Potchefstroom campus).

Organisational procedures

Ten to twenty participants visited the Metabolic Unit facilities on the Potchefstroom Campus of the North-West University daily for a period of seven weeks. The Metabolic Unit consists of 10 bedrooms, two bathrooms, a living room and kitchen. The participants arrived at 07:00 each morning and were accompanied by field workers who explained the setup.

Throughout the morning, the participants completed basic health, demographic and lifestyle questionnaires where they were asked to indicate alcohol and smoking habits. After the questionnaires were completed, blood sampling was done and cardiovascular measurements were taken. The participants received breakfast and a small financial compensation. They also received a report containing their individual health information and if any abnormalities were identified they were referred to their local clinic, hospital or physician.

Anthropometric measurements

Height, body mass, waist – and hip circumferences of each participant were taken in triplicate following standard procedures.[30] We used the Invicta Stadiometer (IP 1465, Leicester, UK) to measure maximum height to the nearest 0.1 cm. Weight was measured to the nearest 0.1 kg using a digital scale (Precision Health Scale, A & D Company, Tokyo, Japan).

Cardiovascular measurements

Systolic and diastolic blood pressure and heart rate were measured after a 10-minute rest in a sitting position, using the OMRON HEM-757 device (OMRON Healthcare, Kyoto, Japan). The appropriate cuff size was used for obese individuals and the blood pressure cuff was always used on the left upper arm. Two measurements were taken with a 5-minute rest interval.

Cardiovascular parameters were obtained using the Finometer^{TM} device (FMS, Finapres Medical Systems, Amsterdam, Netherlands). This involved a 5-minute continuous recording of each subject's cardiovascular measurements under resting, yet awake conditions. After the first two minutes the upper arm pressure was calibrated with the finger pressure for each individual subject (i.e. return-to-flow systolic calibration). The last two minutes of each recording were used to calculate the average of the cardiovascular variables, namely stroke volume, cardiac output, total peripheral resistance and Windkessel arterial compliance.

Biochemical analyses

Fasting blood glucose was directly measured in the Metabolic Unit by a nurse using an enzymatic method to screen for diabetes mellitus (LifeScan SureStep Blood Glucose Monitoring System, LifeScan Inc., Milputas, Ca 9535, USA). A fasting blood sample was taken from the antebrachial vein using a sterile winged infusion set and syringes. Standard methods were used to prepare plasma and serum samples, which were stored at -80ºC until analyses. Immediately after blood sampling, human immunodeficiency virus (HIV) status was determined with rapid tests. Serum was used for testing with the First Response test (PMC Medical, Daman, India) and was repeated with the Pareeshak test (BHAT Bio-tech, Bangalore, India) for confirmation.

Serum blood glucose, total cholesterol, gamma glutamyl transferase and high-sensitivity C-reactive protein (hs-CRP) were determined later in the laboratory using the Konelab 20i auto-analyser (Thermo Fisher Scientific Oy, Vantaa, Finland).

Serum ROS was determined with a high-throughput spectrophotometric assay, with 1 unit equalling 1 mg/liter H_2O_2.[31] Human $VEGF_{165}$ and human Ang-2 were determined with enzyme-linked immunosorbant assays (ELISA) (R&D Systems, Inc., Minneapolis, MN 55413, USA, catalog numbers DANG20 and DVE00, respectively).

Statistical analyses

For database management and statistical analyses we used Statistica software v10 (Statsoft, Inc., 1984-2011). Means were compared by a standard independent T-test, whereas the Chi-square test (χ^2) was done to compare proportions. The biochemical measurements (except for ROS and total cholesterol) were normalised by means of logarithmic transformation. The geometric mean and the 5^{th} and 95^{th} percentile intervals represented the central tendency and spread of these variables. We also tested interactions with ethnicity and gender for the associations of ROS with VEGF and Ang-2 by introducing appropriate interaction terms. Single regression analyses were performed to determine associations between ROS and systolic blood pressure, diastolic blood pressure, VEGF, as well as Ang-2. Quartiles of ROS were plotted against VEGF and Ang-2 in the different ethnic and gender groups, whilst adjusting for age and body mass index. Multiple regression analyses were performed with VEGF or Ang-2 as dependent variables and reactive oxygen species as the main independent variable. Covariates included age, body mass index, total cholesterol, serum glucose, C-reactive protein, physical activity, gamma glutamyl transferase, current smoking and use of anti-hypertensive medication.

Results

The characteristics of the African and Caucasian groups are presented in Table 1. While the two groups were of similar age ($p=0.44$) the Caucasians had a higher body mass index accompanied by higher serum glucose and total cholesterol levels ($p<0.001$) compared to the Africans. In addition, the African participants had significantly higher levels of gamma glutamyl transferase ($p<0.001$) and included more smokers ($p<0.001$) and hypertensive individuals ($p<0.001$). Anti-hypertensive medication intake was significantly higher in the Caucasians ($p<0.001$). The Africans had higher blood pressures ($p<0.001$), as well as a higher heart rate ($p=0.01$). They also had significantly higher levels of ROS ($p=0.002$), VEGF ($p=0.002$) and Ang-2 ($p<0.001$).

Significant interactions were found with ethnicity and gender for the associations of ROS with VEGF and Ang-2, therefore we were able to compare the ethnic and gender groups. Single regression analyses were performed to explore the associations of VEGF, Ang-2 and ROS with cardiovascular measurements (Table 2). There were no significant correlations of VEGF or Ang-2 with any cardiovascular measurements in the African and Caucasian groups, except for correlations between Cwk and VEGF ($r=-0.23$; $p=0.01$) and Cwk and Ang-2 ($r=-0.32$; $p<0.001$) in the African men. ROS correlated significantly with the angiogenic growth factors in both groups of men, being stronger in the African men (both $r=0.33$; $p<0.001$) and only present between VEGF and ROS in the Caucasian men ($r=0.16$; $p=0.04$). ROS also correlated significantly with Ang-2 in the African women ($r=0.26$; $p=0.003$). In addition to this, ROS associated significantly, but weakly with diastolic blood pressure in the Caucasian women ($r=0.15$; $p=0.03$).

Table 1: Characteristics of the African and Caucasian groups.

	Africans	Caucasians	P
N	262	364	
Age (years)	41.4 ± 13.2	40.6 ± 12.9	0.44
Women, n (%)	133 (50.7)	203 (55.7)	0.22
Body mass index (kg/m^2)	23.9 ± 7.2	27.7 ± 5.8	<0.001
Biochemical measurements			
ROS (units)	90.8 ± 24.2	84.8 ± 22.8	0.002
VEGF (pg/mL)	233 (54; 870)	189 (50; 612)	0.002
Ang-2 (pg/mL)	2337 (1233; 4661)	2015 (1230; 3576)	<0.001
Serum glucose (mmol/L)	5.10 (4.02; 6.63)	5.47 (4.44; 7.28)	<0.001
Total cholesterol (mmol/L)	4.47 ± 1.10	5.85 ± 1.43	<0.001
C-reactive protein (mg/L)	2.28 (0.04; 24.53)	1.29 (0.04; 13.59)	<0.001
Gamma glutamyl transferase (U/L)	64.3 (17.9; 420.9)	30.7 (14.4; 85.4)	<0.001
Cardiovascular measurements			
SBP (mmHg)	127 ± 23	119 ± 16	<0.001
DBP (mmHg)	84 ± 14	78 ± 10	<0.001
MAP (mmHg)	105 ± 13	99 ± 10	<0.001
Heart rate (bpm)	70 ± 14	67 ± 9	0.01
TPR (mmHg.s/ml^{-1})	1.38 ± 0.58	1.06 ± 0.33	<0.001
Cwk (ml/mmHg)	1.61 ± 0.50	2.10 ± 0.57	<0.001
Lifestyle and pre-existing conditions			
Baecke total physical activity index	7.77 ± 1.37	7.53 ± 1.43	0.04
Smoking, n (%)	160 (61.1)	52 (14.3)	<0.001
Hypertensive, n (%)	94 (35.9)	49 (13.5)	<0.001
Diabetics, n (%)	10 (3.8)	8 (2.2)	0.23
Medication intake			
Contraception pill, n/total women	4/133	36/203	<0.001
Contraception-DMPA, n/total women	20/133	6/203	<0.001
Anti-hypertensive medication, n (%)	2 (0.8)	71 (19.5)	<0.001
Anti-diabetic medication, n (%)	1 (0.4)	1 (0.3)	0.81

Values are arithmetic mean ± SD, geometric mean (5^{th} to 95^{th} percentile interval), or number of men (%). ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; Ang-2, angiopoietin-2; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; TPR, total peripheral resistance; Cwk, Windkessel arterial compliance; DMPA, depot medroxyprogesterone acetate.

Table 2: Unadjusted associations of markers of angiogenesis and ROS with cardiovascular measurements.

	Vascular endothelial growth factor			
	African		Caucasian	
	Men	Women	Men	Women
SBP (mmHg)	r=0.08; p=0.38	r=0.13; p=0.16	r=0.12; p=0.14	r=0.05; p=0.48
DBP (mmHg)	r=-0.01; p=0.99	r=0.12; p=0.17	r=0.14; p=0.08	r=0.08; p=0.27
MAP (mmHg)	r=0.04; p=0.67	r=0.13; p=0.15	r=0.14; p=0.08	r=0.07; p=0.35
TPR (mmHg.s/ml^{-1})	r=0.03; p=0.74	r=0.07; p=0.46	r=-0.06; p=0.45	r=0.06; p=0.37
Cwk (ml/mmHg)	r=-0.23; p=0.01	r=-0.05; p=0.56	r=-0.07; p=0.35	r=-0.10; p=0.15
	Angiopoietin-2			
SBP (mmHg)	r=0.04; p=0.70	r=0.19; p=0.04	r=0.13; p=0.14	r=0.09; p=0.22
DBP (mmHg)	r=-0.04; p=0.65	r=0.09; p=0.32	r=0.14; p=0.11	r=0.10; p=0.19
MAP (mmHg)	r=-0.00; p=0.98	r=0.15; p=0.10	r=0.15; p=0.10	r=0.10; p=0.18
TPR (mmHg.s/ml^{-1})	r=0.02; p=0.80	r=-0.02; p=0.79	r=-0.11; p=0.21	r=0.03; p=0.73
Cwk (ml/mmHg)	r=-0.32; p<0.001	r=-0.05; p=0.60	r=0.04; p=0.68	r=-0.04; p=0.64
	Reactive oxygen species			
SBP (mmHg)	r=0.09; p=0.32	r=-0.15; p=0.08	r=0.07; p=0.37	r=0.08; p=0.29
DBP (mmHg)	r=0.08; p=0.37	r=-0.17; p=0.06	r=0.08; p=0.32	r=0.15; p=0.03
MAP (mmHg)	r=0.09; p=0.33	r=-0.16; p=0.06	r=0.08; p=0.31	r=0.12; p=0.09
TPR (mmHg.s/ml^{-1})	r=-0.01; p=0.92	r=-0.17; p=0.05	r=-0.06; p=0.45	r=-0.16; p=0.03
Cwk (ml/mmHg)	r=-0.26; p=0.003	r=0.22; p=0.01	r=-0.03; p=0.75	r=0.04; p=0.58
VEGF (pg/mL)	r=0.33; p<0.001	r=0.03; p=0.71	r=0.16; p=0.04	r=0.06; p=0.42
Ang-2 (pg/mL)	r=0.33; p<0.001	r=0.26; p=0.003	r=0.16; p=0.07	r=-0.04; p=0.57

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; TPR, total peripheral resistance; Cwk, Windkessel arterial compliance; VEGF, vascular endothelial growth factor; Ang-2, angiopoietin-2.

In Figure 1 we plotted VEGF and Ang-2 by quartiles of ROS and adjusted for age and body mass index. In all instances African men and women showed significant associations of VEGF and Ang-2 with ROS (p for trend ≤ 0.025), except for the association between VEGF and ROS in African women (p for trend=0.80). Conversely, no significant associations were evident for both Caucasian gender groups. The weak associations between ROS and blood pressure were lost after adjusting for age and body mass index (results not shown).

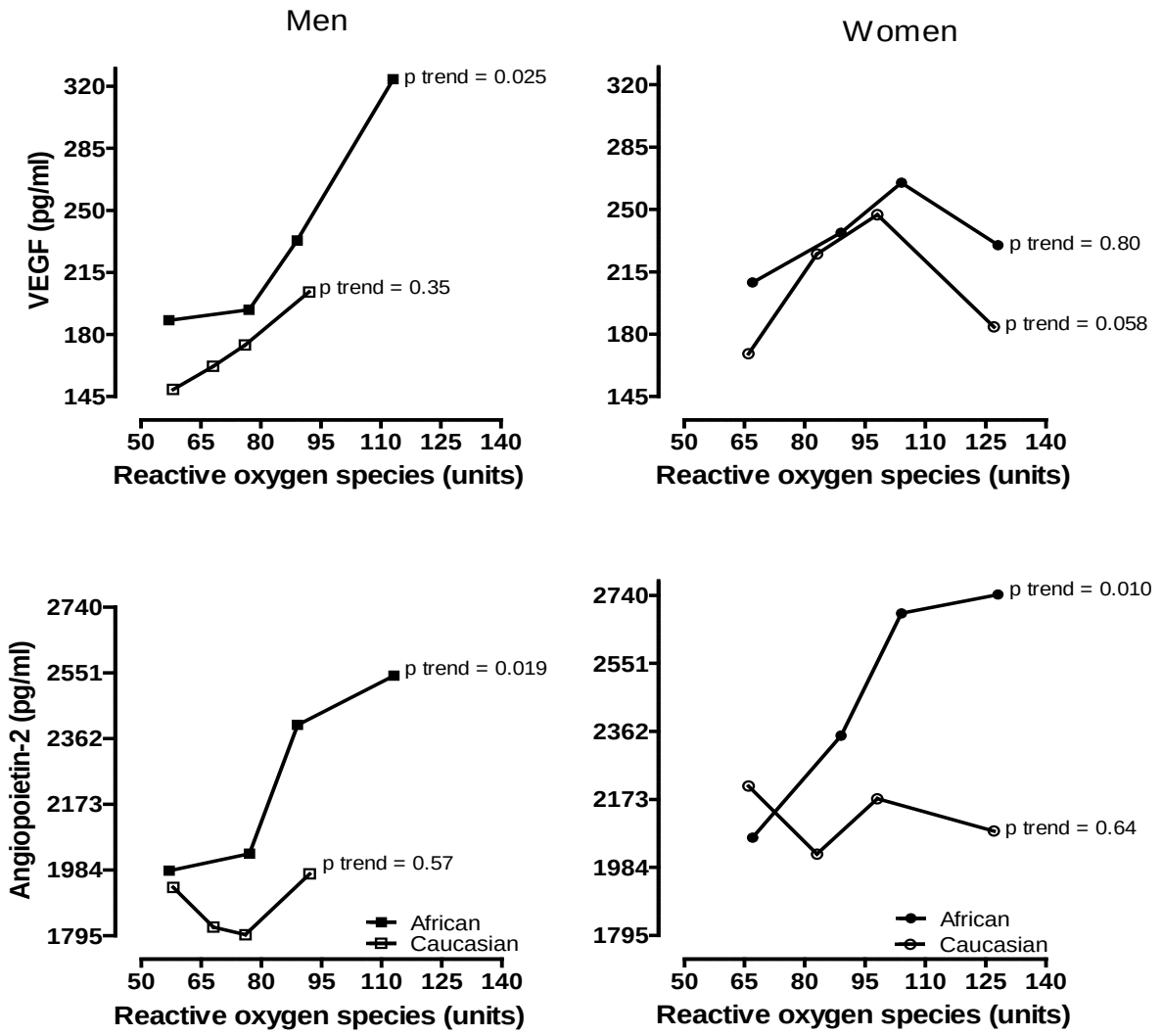

Figure 1: Associations of VEGF and Ang-2 with reactive oxygen species, adjusted for age and body mass index.

VEGF, vascular endothelial growth factor.

To further investigate the significant associations found only in the African group, we performed multiple regression analyses with blood pressure or markers of angiogenesis as dependent variables (Table 3). After full adjustment (age, body mass index, total cholesterol, serum glucose, C-reactive protein, physical activity, gamma glutamyl transferase, current smoking and use of anti-hypertensive medication), the associations of both the angiogenic growth factors with ROS in the African men (both p=0.014) and

Ang-2 with ROS in the African women (p=0.025) were confirmed. No associations were found between the angiogenic growth factors or ROS with blood pressure.

Sensitivity analyses

Due to the known influence of contraceptive medication on ROS,[32] we repeated the multiple regression analyses in women, whilst adding contraceptive medication as a covariate. This did not have any effect on the associations between the angiogenic growth factors and ROS (results not shown).

Table 3: Multiple regression analyses with markers of angiogenesis as dependent variables in the African men and women.

	Independent variable							
	African men				African women			
	Vascular endothelial growth factor							
Dependent variable	R^2	Adj R^2	Std β (95% CI)	p	R^2	Adj R^2	Std β (95% CI)	p
SBP (mmHg)	0.393	0.343	-0.014 (-0.171 to 0.142)	0.86	0.358	0.302	0.077 (-0.082 to 0.236)	0.34
DBP (mmHg)	0.414	0.366	-0.096 (-0.251 to 0.058)	0.22	0.244	0.178	0.124 (-0.048 to 0.297)	0.16
MAP (mmHg)	0.422	0.375	-0.057 (-0.210 to 0.096)	0.47	0.311	0.251	0.103 (-0.061 to 0.267)	0.22
	Angiopoietin-2							
SBP (mmHg)	0.394	0.346	-0.038 (-0.212 to 0.137)	0.67	0.369	0.314	0.136 (-0.020 to 0.291)	0.089
DBP (mmHg)	0.416	0.370	-0.125 (-0.296 to .046)	0.16	0.234	0.167	0.059 (-0.113 to 0.230)	0.50
MAP (mmHg)	0.424	0.378	-0.083 (-0.253 to 0.087)	0.34	0.311	0.251	0.103 (-0.059 to 0.266)	0.21
	Reactive oxygen species							
SBP (mmHg)	0.394	0.346	-0.033 (-0.192 to 0.125)	0.68	0.367	0.312	-0.134 (-0.297 to 0.030)	0.11
DBP (mmHg)	0.406	0.359	-0.019 (-0.175 to 0.138)	0.82	0.253	0.188	-0.167 (-0.345 to 0.011)	0.068
MAP (mmHg)	0.420	0.374	-0.027 (-0.182 to 0.128)	0.73	0.321	0.262	-0.155 (-0.325 to 0.015)	0.075
VEGF (pg/mL)	0.184	0.118	0.235 (0.049 to 0.421)	0.014	0.157	0.084	-0.105 (-0.294 to 0.084)	0.28
Ang-2 (pg/mL)	0.358	0.307	0.207 (0.044 to 0.369)	0.014	0.165	0.092	0.218 (0.030 to 0.406)	0.025

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; VEGF, vascular endothelial growth factor; Ang-2, angiopoietin-2; ROS, reactive oxygen species. Adjusted for age, body mass index, total cholesterol, serum glucose, C-reactive protein, physical activity, gamma glutamyl transferase, current smoking and use of anti-hypertensive medication.

Discussion

We investigated the relationships between the angiogenic growth factors (VEGF and Ang-2) and oxidative stress in Africans and Caucasians, as well as the associations of these markers with cardiovascular measures. While no association existed with cardiovascular measurements, Ang-2 was significantly and independently associated with ROS in African men and women. African men also showed a significant association between VEGF and ROS, whereas no significant associations were found in the Caucasian gender groups.

African Americans have previously shown higher levels of oxidative stress by displaying higher levels of hydrogen peroxide and NADPH-derived oxidative stress, as well as increased antioxidant activity compared to Caucasians.[29] Literature regarding the mechanisms behind this ethnic difference in oxidative stress, as well as on levels of angiogenic growth factors in Africans are limited. We can, however, confirm that black South Africans not only presented a more vulnerable cardiovascular profile, but also higher levels of oxidative stress and angiogenic activity compared to Caucasians.

The most prominent finding of our study was the strong link between angiogenic growth factors and oxidative stress only in black South Africans. A possible explanation for the significant correlations is the influence of nitric oxide (NO). ROS decreases the bioavailability of NO through the formation of peroxynitrite.[7,33] Oxidative stress also increases asymmetric dimethylarginine (ADMA) concentrations.[34] ADMA is a competing factor to L-arginine for NO substrate and contribute to a decrease in NO bioavailability.[35] Melikian et al.[27] found ADMA levels to be higher in African men. In addition, our group previously found in this study no significant difference in ADMA levels between African and Caucasian men,[36] however, L-arginine levels were significantly lower in African men.[37] These findings could therefore suggest a decreased bioavailability of NO in Africans, resulting in decreased endothelial function compared to Caucasians.[27,29,34] VEGF, on the other hand, causes an increase in NO syntheses. However, NO has a negative feedback effect on VEGF.[10,33] Therefore, decreased levels of NO may result in increased levels of VEGF in the African men.

It has been shown that oxidative stress has an essential role to play in angiogenesis either causing angiogenesis as a response to angiogenic growth factors, such as VEGF and Ang-1, or by stimulating angiogenic growth factors, such as VEGF and Ang-2, leading to endothelial cell migration and proliferation.[7,8,11] We cannot infer any causality; however, we are tempted to hypothesise the higher levels of VEGF and Ang-2 in the African participants to be driven by oxidative stress. This is supported by other studies that show an increase in oxidative stress leads to an increase in angiogenic growth factors.[38-40] Adding to this, the African participants showed higher levels of serum glucose ($p<0.001$), C-reactive protein ($p<0.001$), gamma glutamyl transferase ($p<0.001$) and a more vulnerable cardiovascular profile. The African group also included more smokers and had the least number of people using anti-hypertensive medication. All of these mentioned characteristics are involved in increasing oxidative stress,[4,5,41-43] possibly leading to an increase in angiogenesis. Angiogenesis and oxidative stress linked significantly in the African men, who also displayed the more vulnerable cardiovascular profile. Therefore, it is uncertain whether this association between angiogenesis and oxidative stress is dependent on ethnicity or whether it is dependent on cardiovascular profile.

Although mean values show that the Africans had higher blood pressure than the Caucasians, it is not yet severely elevated. However based on the known association of VEGF, Ang-2 and oxidative stress in cardiovascular disease,[1-3,10,17,21-23] it is not unrealistic to expect significant elevations in blood pressure over time in this particular group of Africans. The independent link between ROS and VEGF found only in African men is supported by the results that this subgroup had the highest blood pressures and lowest L-arginine levels[37] of all ethnic and gender groups.

We also aimed to investigate the association of cardiovascular measurements with oxidative stress and angiogenic growth factors. Surprisingly, we found no associations of blood pressure with ROS or angiogenic growth factors in the Africans or Caucasians. Oxidative stress is known to be involved in the pathogenesis of hypertension through an increase in ROS and a decrease in antioxidant activity.[2,4] It is unclear whether oxidative stress is a cause or a result of hypertension.[45] However, the association between oxidative stress and hypertension could depend on the type of marker of oxidative stress used.[45] Keaney et al.[41] used the oxidative stress marker creatinine-indexed urinary 8-epi-

$PGF_{2\alpha}$ and also found no association with hypertension. When comparing their data to previous animal studies they also suggested that the association between hypertension and oxidative stress might only apply in certain hypertension states.[41]

The lack of association in our study between blood pressure and angiogenesis is also unexpected, since VEGF and Ang-2 have also shown to be increased in hypertension.[8,19,24,44] VEGF is a regulator of endothelial and vascular function as well as blood pressure and deregulation of this growth factor may lead to its increase in blood pressure.[46-49] Detailed mechanisms explaining the increase in both growth factors during this disease are lacking. It is also unknown if the increase in angiogenic growth factors in hypertension is a cause or an effect and this increase may only reflect endothelial damage caused by hypertension.[26,46]

The intricate relationship between blood pressure, oxidative stress and angiogenesis are clearly not well understood. But it is likely that these angiogenic growth factors are increased in the Africans due to vascular remodelling, since VEGF and Ang-2 are linked to vascular remodelling.[44,50-52] Cardiovascular diseases are also closely linked to vascular remodelling through oxidative stress.[53] Due to the more vulnerable cardiovascular profile of the African group and higher ROS compared to Caucasians, it is possible that Africans may have increased vascular remodelling accompanied by increased angiogenic growth factors.

Unfortunately this study could not be concluded without limitations. We could have included a more complete angiogenic and oxidative stress profile by including additional markers. More sensitive vascular function measurements as well as 24-h blood pressure measurements could have yielded different results. This study was performed on 626 volunteers; therefore the findings cannot be extrapolated to the general population. Although our results remained consistent after adjusting for confounders, it is possible that the associations may have been influenced by unknown confounders. Causality cannot be inferred, as this was a cross-sectional study. However, this study was well-designed and performed under controlled conditions.

To conclude, we found in our study that the angiogenic growth factor, Ang-2, was significantly associated with oxidative stress only in the African men and women. Additionally, VEGF was significantly linked to oxidative stress only in the African men. These associations were absent in the Caucasian men and women. It therefore seems as if angiogenesis and oxidative stress are better associated in the Africans compared to the Caucasians, possibly adding to the existing high risk of cardiovascular disease in this population.

Table 4: Summary table.

What is known about the topic:	**What this study adds:**
• Oxidative stress levels are higher in African Americans. [29] • Oxidative stress induces angiogenesis either through stimulation of angiogenic growth factors or in response to angiogenic growth factors.[7,8]	• VEGF-, Ang-2 levels and oxidative stress are higher in the African group, compared to Caucasians. • Ang-2 was significantly associated with oxidative stress only in Africans. • VEGF was linked to oxidative stress only in African men. • The above mentioned relationships were absent in Caucasians.

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Chapter Four

Summary of the main findings and recommendations for future research

Introduction

In this chapter the main findings of the study will be discussed and then compared to existing literature, after which a conclusion will be made and recommendations will be proposed for future research regarding angiogenesis and oxidative stress in the African population.

Summary of the main findings

Our first hypothesis stated that the cardiovascular measurements, reactive oxygen species and angiogenic growth factors are elevated in Africans when compared to Caucasians. This hypothesis can be accepted as we found that the African group had elevated levels of both vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang-2), as well as elevated levels of reactive oxygen species (ROS) when compared to the Caucasians. They also displayed a poorer cardiovascular profile and included more smokers and the least number of people using anti-hypertensive medication.

We also aimed to determine whether relationships exist between angiogenic growth factors and oxidative stress in African and Caucasian gender groups as set out in the second hypothesis: oxidative stress show strong associations with angiogenic growth factors in Africans compared to Caucasians. We found that Ang-2 was significantly and independently associated with oxidative stress in African men and women and VEGF significantly associated with oxidative stress only in the African men. These significant associations were absent in the Caucasian gender groups. The second hypothesis can therefore be partially accepted, due to the significant associations found in the Africans.

Surprisingly, we found no associations between the angiogenic growth factors, VEGF and Ang-2, or ROS with blood pressure in neither the Africans nor the Caucasians. The third hypothesis is therefore rejected which stated that cardiovascular measurements are adversely associated with oxidative stress and angiogenic growth factors in Africans and Caucasians, but these associations are stronger in Africans.

Discussion of the main findings

In the present study our main finding was that the angiogenic growth factor, Ang-2, was associated with oxidative stress in African men and women and VEGF, the other angiogenic factor, was associated with oxidative stress only in the African men. Oxidative stress causes angiogenesis as a response to angiogenic growth factors, such as VEGF and Ang-1 and again causes angiogenesis through the stimulation of the angiogenic growth factors, VEGF and Ang-2.[1-3] We proposed that the higher levels of VEGF and Ang-2 in the Africans could be driven by oxidative stress. This is supported by our findings, as well as previous studies.[4-6] Sihvo et al.[5] found a simultaneous increase in oxidative stress and angiogenesis. They suggested oxidative stress to be the pathway for the onset and process of angiogenesis.[5] Adding to this, Khatri et al.[4] found that smooth muscle cells exhibiting oxidative stress expressed high levels of VEGF.[4] An increase in pancreatic angiogenesis due to an accumulation of ROS in pancreatic β-cells was found by Laurent et al.[6] They also found that an increase in H_2O_2 leads to the stabilisation of hypoxia inducible factor-1α in β-islets, thereby stimulating VEGF-A.[6]

A possible mechanism for the association between the angiogenic growth factors and oxidative stress in the African group involves nitric oxide (NO), which has a negative feedback effect on VEGF.[7,8] However, NO is decreased in Africans as we previously found L-arginine levels, which is the precursors of NO, to be significantly lower in the African men.[9,10] In addition to this, ROS as well as Ang-2 (which are both increased in our African group) have shown to decrease NO.[1,8,11] Therefore, the decreased levels of NO could result in increased levels of VEGF in the African men.

We found that the African participants in our study presented a more vulnerable cardiovascular profile compared to the Caucasians, mainly elevated levels of oxidative stress (ROS), angiogenic growth factors (VEGF and Ang-2) and blood pressure. In support of this, African Americans have previously shown increased levels of oxidative stress compared to Caucasians.[12] Additionally African Americans and black South Africans show increased risk for cardiovascular diseases, particularly hypertension and stroke.[13-17] Since limited information is available regarding angiogenic activity in the African population, our results present some of the first findings on the link between

oxidative stress and angiogenesis in a African population with an elevated cardiovascular risk profile.

Surprisingly, we found no associations between the angiogenic growth factors or ROS with blood pressure. This contradicts, but is also supported by previous studies. It has been found that oxidative stress is involved in the pathogenesis of hypertension.[18,19] A wide variety of mechanisms exist by which oxidative stress may lead to hypertension.[20] One possible mechanism is the NADPH oxidase pathway. The increase in angiotensin II during hypertension causes an increase in NADPH oxidase through the stimulation of angiotensin I receptor.[18,21] There is however uncertainty whether oxidative stress is a cause or a result of hypertension, however the latter is favoured by Grossman.[20] Our lack of association may be supported by the conclusion that the association between oxidative stress and hypertension is dependent on the marker of oxidative stress used.[20] Keaney et al.[22] used the oxidative stress marker, creatinine-indexed urinary 8-epi-$PGF_{2\alpha}$, and also found no association between oxidative stress and hypertension. He suggested that the association might only apply in certain hypertension states.[22]

The angiogenic growth factors, VEGF and Ang-2, have also shown to be increased in hypertension,[3,23-25] thus opposing our lack of association with blood pressure in both ethnic groups. It is uncertain whether the increase in angiogenic growth factors in hypertension is a cause or an effect as it was proposed that it may only reflect endothelial damage caused by hypertension.[26,27] We suggested the increase in angiogenic growth factors in our black participants to be due to vascular remodelling as both VEGF and Ang-2 are increased in vascular remodelling.[24,28-30] Cardiovascular diseases are also closely linked to vascular remodelling.[31]

Chance, bias and confounding

Before a final conclusion can be drawn and possible recommendations be proposed, it is critical to reflect on some of the important factors, such as methodological issues that might have caused weaknesses in this study and influenced the outcomes.

The number of subjects used in this study is questionable. After excluding pregnant and lactating individuals and persons with human immunodeficiency virus, a total of 626 participants remained. It is possible that the results would have been more reliable if

larger subject groups were used. However, when comparing our sample size to those used in the literature, it seems to be larger than most other studies.[2,5,27,32-38]

Information as well as selection bias exist. A possible false high blood pressure may have been presented by some subjects due to "the white coat effect." This study also made use of an availability sample. Our study group consisted mostly of poor and unemployed black participants, whereas the Caucasians were mostly of higher socio-economic class. It is also possible that there are other unknown factors that influenced the variables under study and may have produced inaccurate results.

Although we adjusted for age, body mass index, total cholesterol, serum glucose, C-reactive protein, physical activity, gamma glutamyl transferase, current smoking and use of anti-hypertensive medication in the multiple regression analyses, it is possible that these confounders may have influenced the results by causing an over- or underestimation of the associations between the angiogenic growth factors and ROS with blood pressure and also the association between these angiogenic growth factors and ROS.

When interpreting the statistical results of this study, we aimed to do so from a physiological standpoint, however keeping in mind that a statistical significance does not necessarily imply a physiological significance and vice versa.

Conclusion

We found that angiogenesis and oxidative stress were strongly associated in Africans, whereas these associations were absent in Caucasians. More specifically, the angiogenic growth factor, Ang-2, was significantly associated with oxidative stress in the African men and women, whereas the other angiogenic marker, VEGF, associated with oxidative stress only in the African men. These associations were not found in the Caucasian gender groups. It is possible that these associations are contributors to the high cardiovascular risk profile of the African population and should be investigated further in prospective and experimental studies.

Recommendations

The authors recommend the following for future studies which may focus on the topic of oxidative stress, angiogenesis and cardiovascular disease in humans:

- The use of a wider variety and more sensitive cardiovascular measurements, for example: flow-mediated dilation and pulse wave velocity for indication of endothelial function and arterial stiffness.
- The use of various additional angiogenic growth markers, for example angiopoietin-1, fibroblast growth factor, transforming growth factor, as well as additional oxidative stress markers.
- The investigation of the influence of a specifically detailed cardiovascular profile on angiogenesis in future studies might lead to novel findings.
- Larger sample size and subject groups selected through random sampling would be nationally representative.
- Longitudinal studies featuring target organ damage, cardiovascular events and mortality are also strongly recommended.

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