Exploring a marker of cardiac fibrosis and its association with soluble uPAR in a bi-ethnic South African population: The SAfrEIC study

CS du Plooy
21247366

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Supervisor:	Prof HW Huisman
Co-supervisor:	Dr R Kruger
Assistant supervisor:	Prof AE Schutte

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Table of content

Table of content (continued)

Contribution of the authors

The relative contributions of each researcher involved in this study are given below:

Ms. CS du Plooy	Responsible for the review of the literature, statistical analysis, design and planning of manuscript, interpretation of results and writing the manuscript.
Prof. HW Huisman	Supervisor. Supervised writing of the manuscript and initial planning and design of the dissertation and manuscript as well as collection of the data.
Dr. R Kruger	Co-supervisor. Supervised writing of the manuscript, initial planning and design of the dissertation and manuscript.
Prof. AE Schutte	Assistant supervisor. Assisted with the writing of the manuscript and design of the dissertation and manuscript as well as the initial design, management and data collection of the SAfrEIC study.

The following is a statement from the co-authors confirming their individual roles in this study and giving their permission that the article may form part of this dissertation.

Prof. HW Huisman

Dr. R Kruger

Prof. AE Schutte

Summary

Exploring a marker of cardiac fibrosis and its association with soluble uPAR in a bi-ethnic South African population: The SAfrEIC study.

Background

Fibulin-1, an extracellular matrix component and mediator in cardiac fibrosis, is expressed in cardiac valves, heart muscles and blood vessels and may contribute to different cardiovascular pathological conditions such as hypertension, aortic valve stenosis, atrial fibrillation and coronary artery disease. The most conspicuous functions of fibulin-1 include cell adhesion and cell migration within the extracellular matrix (ECM). This was found to reflect vascular dysfunction contributing to the development of fibrosis in the myocardium by means of changes in the ECM, possibly as a result of inflammation.

Inflammatory mediators such as C-reactive protein (CRP) and albumin have been investigated over the years for the role they play in the inflammatory processes. However, one inflammatory mediator, soluble urokinase-type plasminogen activator receptor (suPAR), only emerged as a potential biomarker in the development of sclerotic disease. SuPAR is a soluble bioactive form of the urokinase-type plasminogen activator receptor (uPAR) secreted by inflammatory cells such as macrophages, endothelial cells and monocytes. The most profound functions of suPAR such as cell migration and cell adhesion contribute to the development of diseases such as infection, autoimmune diseases, cancer and atherosclerosis.

Motivation and aim

This study was motivated by an awareness of the limited data on the potential link between fibulin-1 and suPAR, along with other markers of inflammation (CRP and albumin). We aimed to compare the levels of a marker of cardiac fibrosis (fibulin-1) and inflammatory mediators (suPAR, CRP and albumin) in African and Caucasian men and women. A second aim was to explore fibulin-1 and its potential association with these inflammatory markers independent of haemodynamic and metabolic risk factors in a bi-ethnic cohort from South Africa.

Methodology

Data from the cross-sectional SAfrEIC study (*South African study regarding the role of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function)* were used, which initially included 756 participants. Our study population comprised 290 Africans (men: n=130; women: n=160) and 343 Caucasians (men: n=160; women: n=183). We excluded HIV-infected participants (n=115) as well as those with missing data (n=8). Traditional cardiovascular measurements together with the relevant biochemical analyses were done. T-tests and Chi-square tests were used to compare means and proportions between groups, respectively. Single and partial correlations were performed to determine the relationship of fibulin-1 with suPAR, CRP and albumin, with adjustments for age. SuPAR, CRP and albumin were divided into tertiles to explore the association with fibulin-1 levels, while adjusting for age, body mass index (BMI) and diastolic blood pressure (DBP) by using analysis of covariance (ANCOVA). Multiple regression analysis was performed to explore independent associations.

Results

Participants were divided into African and Caucasian men and women due to significant interactions of the main effects of ethnicity and gender on the association of fibulin-1 with suPAR (ethnicity: $F(633)=7.29$; $p<0.001$ and gender: $F(633)=7.99$; $p<0.001$). Fibulin-1 levels were higher in African men ($p=0.010$), whereas CRP was higher in African women ($p<0.001$) compared to their Caucasian counterparts. In both gender groups suPAR levels were higher and albumin lower in Africans compared to Caucasians ($p<0.006$). In single regression analyses, a positive correlation existed between fibulin-1 and suPAR in African ($r=0.19$; $p=0.028$) and Caucasian men ($r=0.37$; $p<0.001$), also in African ($r=0.193$; $p=0.028$) and Caucasian women ($r=0.14$; $p=0.036$). After adjustments were applied for age, this correlation remained in African ($r=0.23$; $p=0.010$) and Caucasian men ($r=0.22$; $p=0.005$) only. An inverse correlation was found between fibulin-1 and albumin in African men ($r=-0.28$; $p=0.002$), but not in Caucasian men ($r=-0.09$; $p=0.245$). No significant correlation was found between fibulin-1 and CRP in any group. Forward stepwise regression analysis was performed in men and the

previous associations between fibulin-1 and suPAR were confirmed in African and Caucasian men; along with the inverse relationship of fibulin-1 with albumin (Adj. R^2=0.217; β=–0.210; p=0.013) in African men only.

Conclusion

Fibulin-1 was positively associated with suPAR in African and Caucasian men, but not in women. We also found fibulin-1 to be negatively associated with albumin in African men only. These results are indicative of the presence of potential subclinical low-grade inflammation as depicted by suPAR within the extracellular matrix. This low-grade inflammation may contribute to the potential onset of cardiac fibrosis or vascular sclerosis among these South African men with lower albumin levels.

Keywords: Fibulin-1, suPAR, cardiac fibrosis, inflammation, extracellular matrix remodelling, African, Caucasian

Afrikaanse titel en opsomming

Ondersoek van 'n merker van hartfibrose en die assosiasie daarvan met oplosbare uPAR in 'n bi-etniese Suid-Afrikaanse populasie: Die SAfrEIC studie.

Agtergrond

Fibulien-1 is 'n ekstrasellulêre matrikskomponent en 'n merker van hartfibrose, wat in hartkleppe, hartspiere en bloedvate voorkom, en tot verskeie kardiovaskulêre patologiese toestande soos hipertensie, aortiese klepstenose, atriale fibrillasie en koronêre hartsiektes kan bydra. Die kenmerkendste funksies van fibulien-1 sluit selverbinding en selmigrasie binne die ekstrasellulêre matriks (ESM) in. Daar is bevind dat vaskulêre disfunksie tot die ontwikkeling van fibrose in the miokardium bydra as gevolg van veranderinge in die ESM moontlik as 'n gevolg van inflammasie.

Navorsing is oor die jare gedoen oor die rol wat inflammatoriese tussengangers soos C-reaktiewe proteien (CRP) en albumien speel in inflammasieprosesse. Daar is bevind dat een inflammatoriese tussenganger, naamlik oplosbare urokinase-tipe plasminogeen aktiveringsreseptor (suPAR), onlangs begin uitstaan het as 'n potensiële tussenganger in die ontwikkeling van sklerotiese prosesse. SuPAR is 'n oplosbare bio-aktiewe vorm van uPAR, wat deur inflammatoriese selle soos makrofages, endoteelselle en monosiete gesekreteer word. Die kenmerkendste funksies van suPAR soos selmigrasie en selverbinding dra by tot die ontwikkeling van siektes soos infeksie, outo-immuunsiektes, kanker en aterosklerose.

Motivering

Die studie is gemotiveer deur die beperkte data wat oor die potensiële verband tussen fibulien-1 en suPAR, asook ander inflammatoriese merkers (CRP en albumien) beskikbaar is. Ons eerste doelwit was om ondersoek in te stel na die vlakke van 'n merker van hartfibrose (fibulien-1) en inflammatoriese tussengangers (suPAR, CRP en albumien) in swart en wit mans en vrouens. 'n Tweede doelwit was om die potensiële verband tussen fibulien-1 en die genoemde

inflammatoriese merkers onafhanklik van hemodinamiese en metaboliese risikofaktore in 'n bi-etniese Suid-Afrikaanse populasie te ondersoek.

Metodologie

Data vanaf die dwarsdeursnit SAfrEIC studie (*South African study regarding the role of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function*) is gebruik waaraan daar oorspronklik 756 proefpersone deelgeneem het. Ons studie het 290 swart (mans: n=130; vrouens: n=160) en 343 wit (mans: n=160; vrouens: n=183) ingesluit. HIV geinfekteerde deelnemers (n=115) sowel as verlore data (n=8) was uitgesluit. Tradisionele kardiovaskulêre metings saam met die relevante biochemiese analises is gedoen. T-toetse is gebruik om gemiddelde tussen die groepe te vergelyk en *Chi*-kwadraat toetse om proporsies tussen die groepe te vergelyk. Enkele korrelasies is gebruik om die ongekorrigeerde korrelasies tussen fibulien-1 en kardiovaskulêre veranderlikes te bepaal. SuPAR, CRP en albumien was verdeel in tertiele om die assosiasie met fibulien-1 vlakke te ondersoek, nadat aangepas is vir ouderdom, liggaamsmassa-indeks en diastoliese bloeddruk deur gebruik te maak van analise van kovariasie (ANCOVA). Meervoudige regressie-analise is uitgevoer om onafhanklike assosiasies te ondersoek.

Resultate

Deelnemers is in swart en wit groepe verdeel op grond van die interaksie op die hoofeffekte van etnisiteit en geslag op die assosiasies van fibulien-1 met suPAR (etnisiteit: $F(633)=7.29$; $p<0.001$ en geslag: $F(633)=7.99$; $p<0.001$). Fibulien-1 vlakke was hoër in swart mans ($p=0.010$), waar CRP hoër was in swart vrouens ($p<0.001$) in vergelyking met hul wit eweknieë. In beide geslagsgroepe was suPAR vlakke hoër en albumien laer in swart in vergelyking met wit ($p<0.006$) proefpersone. In enkele regressie-analises het 'n positiewe assosiasie tussen fibulin-1 en suPAR ontstaan in swart ($r=0.19$; $p=0.028$) en wit ($r=0.37$; $p<0.001$) mans, asook in swart ($r=0.193$; $p=0.028$) en wit vrouens ($r=0.14$; $p=0.036$). Na aanpassings vir ouderdom, is fibulien-1 positief geassosieer met suPAR slegs in swart ($r=0.23$; $p=0.010$) en wit ($r=0.22$; $p=0.005$) mans. 'n Omgekeerde korrelasie bestaan tussen fibulien-1 en albumien in swart mans ($r=-0.28$;

p=0.002), maar nie in wit mans (r=–0.09; p=0.245) nie. Geen korrelasie is tussen fibulin-1 en CRP in enige van die groepe gevind nie. Nadat voorwaartse stapsgewyse regressie-analises in mans uitgevoer is, is die vorige assosiasies tussen fibulien-1 en suPAR bevestig, asook fibulien-1 se omgekeerde verhouding met albumin (p≤0.05) slegs in die swart mans.

Gevolgtrekking

Fibulien-1 is positief geassosieer met suPAR slegs in swart en wit mans, maar nie in vrouens nie. Ons het ook gevind dat fibulien-1 negatief geassosieer is met albumien slegs in swart mans. Hierdie resultate is beduidend van die teenwoordigheid van potensiële subkliniese lae-graadse inflammasie soos voorgestel deur suPAR binne die ekstrasellulêre matriks. Hierdie lae-graadse inflammasie kan moontlik bydra tot die potensiële ontstaan van hartfibrose of vaskulêre sklerose in hierdie Suid-Afrikaanse mans met laer albumien vlakke.

Sleutelwoorde: Fibulien-1, suPAR, hartfibrose, inflammasie, ekstrasellulêre matriks hermodellering, swart, wit

Preface

This dissertation is submitted for the degree Master of Science in Physiology at the Potchefstroom Campus of the North-West University. The format of this dissertation complies with the prescribed article format as approved by the North-West University. This dissertation consists of a manuscript ready for submission to an international peer-reviewed journal. Chapter 1 provides an introduction and motivation regarding the study. A detailed literature overview related to the topic as well as the aims and hypotheses are discussed in Chapter 2. The manuscript (Chapter 3) consists of the abstract, introduction, methodology, results and discussion of the study which will be submitted for publication to the journal *Atherosclerosis*. Chapter 4 is a critical summary of the results, providing final conclusions as well as recommendations. Appropriate references are provided at the end of each chapter according to the style as described by the journal.

Outline of the dissertation

The outline of the study is as follows:

Chapter 1: Introduction and motivation.

Chapter 2: Literature overview, aims and hypotheses.

Chapter 3: Manuscript – *Exploring a marker of cardiac fibrosis and its association with soluble uPAR in a bi-ethnic South African population: The SAfrEIC study.*

Chapter 4: Final conclusions and recommendations.

List of tables

Chapter 3

List of figures

Chapter 3

List of abbreviations

ANCOVA	=	Analysis of covariance
BMI	=	Body mass index
BNP	=	B-type natriuretic peptide
CRP	=	C-reactive protein
CVD	=	Cardiovascular disease
DBP	=	Diastolic blood pressure
ECM	=	Extracellular matrix
ELISA	=	Enzyme-linked immunosorbant assay
GPI	=	Glycosyl phosphatidyl inositol
HIV	=	Human immunodeficiency virus
ICAM-1	=	Intracellular adhesion molecule-1
IL-6	=	Interleukin-6
LDL	=	Low-density lipoprotein
Lp(a)	=	Lipoprotein(a)
MMPs	=	Matrix metalloproteinases
NO	=	Nitric oxide
NT-proBNP	=	N-terminal prohormone-B-type natriuretic peptide
PAI	=	Plasminogen activator inhibitor
ROS	=	Reactive oxygen species
SAfrEIC	=	South African study regarding the role of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function
SBP	=	Systolic blood pressure
SMC	=	Smooth muscle cells
SuPAR	=	Soluble urokinase-type plasminogen activator receptor
uPA	=	Urokinase-type plasminogen activator
uPAR	=	Urokinase-type plasminogen activator receptor
VCAM-1	=	Vascular cell adhesion molecule-1
WHO	=	World Health Organization
γ-GT	=	Gamma glutamyl transferase

Chapter 1

Introduction and motivation

1.1. Introduction and motivation

Cardiovascular diseases (CVDs) are currently a major cause of death globally [1,2]. Many of these diseases (coronary artery disease, hypertension, atherosclerosis and cardiac fibrosis) have an undertone of extracellular matrix (ECM) remodelling in the form of fibrosis or sclerosis [1,3].

Cardiac fibrosis can be defined as a consequence of remodelling processes initiated by pathological events such as proliferation of interstitial fibroblasts and biosynthesis of ECM components in the wall of the ventricles of the heart, which are associated with a variety of cardiovascular disorders [4-6]. This may eventually lead to myocardial stiffness and ventricular dysfunction [4-6]. Two biomarkers, namely fibulin-1 and soluble urokinase-type plasminogen activator receptor (suPAR), emerged as potential mediators in the development and progression of fibrotic and sclerotic processes [6-15]. In addition, the well-known acute phase protein, C-reactive protein (CRP) and the most abundant protein in plasma, albumin, are principally used as biomarkers of inflammation involved in vascular dysfunction and the development of atherosclerosis [16,17].

Fibulin-1 is expressed in the cardiac septa, valves and blood vessels [7,9,13,18] and may contribute to different cardiovascular pathological conditions such as cardiac fibrosis [10]. SuPAR on the other hand, is the soluble form of urokinase-type plasminogen activator receptor (uPAR), released from activated T-lymphocytes, monocytes, endothelial cells, fibroblasts and smooth muscle cells (SMC) [12,19-21]. SuPAR is also released from inflammatory cells, and play a major role in inflammation related to atherosclerotic plaque progression [14]. Fibulin-1 and suPAR share a function in the modulation of cell adhesion [15,19-22]. Although they have this role in common, they function in separate pathways that are connected by its collective purpose of ECM changes.

Studies done in European and American countries have partly investigated fibulin-1 and its role in cardiac fibrosis and ECM remodelling [13]; however, less is known about South African

populations apart from two studies [15,23]. Studies have indicated that Africans are subjected to early changes within the vasculature that elevate their risk for the development of CVD [15,24,25]. In terms of gender, CVD presents itself more profoundly in men [15,24,25]. The limited data on the potential link between fibulin-1 and its association with inflammatory markers, suPAR, CRP and albumin, independent of other haemodynamic and metabolic component in a bi-ethnic cohort from South Africa, motivated this study.

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Chapter 2

Literature study, aims and hypotheses

2.1. General introduction

In this chapter a detailed literature overview related to fibulin-1 and suPAR and the association of these mediators with inflammation and inflammatory markers (such as albumin and CRP) is provided. The literature review includes the role that inflammatory process, inflammatory markers and ECM proteins play in contributing to the development of CVD. The aims and hypotheses of this study will follow the literature study.

Africans are at higher risk for developing CVD attributable to early changes within the vasculature due to lifestyle (smoking, alcohol overuse and unhealthy diet) and inherent (stroke) risk factors [1,2]. Over a sustained period of time, adverse lifestyle choices may alter normal physiology including the structure and function of the cardiovascular and metabolic systems [3]. The early deterioration of the endothelium, provoked by lifestyle factors, elevated blood pressure and dyslipidemia is usually concurrent with the development of atherosclerotic plaque and sclerotic lesions [4-7].

2.2. The inflammatory process in the development of cardiovascular diseases

The vascular endothelium is important for the production of vasoactive compounds such as endothelin, angiotensin, cyclic compound and the secretion of nitric oxide (NO) to prevent the deposition of platelets into the intima [8-11]. Endothelial cells are also responsible for the maintenance of vascular homeostasis and the regulation of vascular tone [12-15].

During inflammation of the endothelium, caused by oxidative stress and cytokines, adhesion molecules (such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecules (ICAMs)), integrins and selectins are expressed that are important for the recruitment of leukocytes to the injured site [13-17]. The integrins facilitate a firmer attachment of the leukocytes and once adhered to the endothelium, the leukocytes penetrate into the intima and become macrophages [16,17]. Proteases such as matrix metalloproteinases (MMPs) are released from the infiltrating immune cells activating resident cells in the tissue interstitium to alter ECM synthesis and cause neutrophils to invade the inflamed area from the blood [18-21].

MMPs selectively cleave to ECM proteins and generate bioactive ECM fragments thereby prolonging inflammation by initiating an increased expression of ICAM-1 on the surface of endothelial cells in the inflamed area [11,22]. Monocytes enter from the blood and enlarge, forming a large number of defensive white blood cells that help remove the cause of inflammation [11,19,22]. The enlargement of local cells and migration of white blood cells toward the inflamed area display an insult on the ECM integrity [18,22]. Sustained inflammatory processes can therefore lead to adverse vascular damage, subsequently contributing to the development of CVD.

2.3. Oxidative stress, antioxidants and atherosclerosis

Oxidative stress reflects the imbalance between antioxidants and reactive oxygen species (ROS) in favour of the latter [16]. During normal physiological conditions the ROS concentration is low and the NO availability is high, maintaining cell activities such as cell growth and cell adaptation responses as well as endothelial-dependent vasodilation [13,14]. Once NO availability is reduced, ROS shifts the vascular tone towards vasoconstriction [16]. Lifestyle factors can cause an increase in ROS generation at the site of inflammation causing endothelial damage and necrotic cell death that contribute to vascular diseases such as atherosclerosis [13,14,16,17].

During atherosclerosis, monocytes attack the endothelium of the arterial lumen leading to the formation of atheromatous plaque in the arterial tunica intima [23,24]. This process is known as atherogenesis [25,26]. Most of these atheromatous plaques consist of excess lipids and disrupted collagen to elastin ratio [25]. Low-density lipoprotein (LDL) particles invade the endothelium and become oxidized [16,26]. Monocytes differentiate into macrophages, which ingest oxidized LDL, turning into large foam cells, leaving behind lesions that appear as a fatty streak [26]. Foam cells eventually die and further propagate the inflammatory process [16]. Smooth muscle cell (SMC) proliferation and migration from the tunica media into the tunica intima cause the formation of a fibrous capsule covering the fatty streak [26]. Intact endothelium

could prevent the proliferation by releasing NO, which does not happen at atherosclerotic sites [26].

Free radicals are involved throughout the atherogenic process, beginning from endothelial dysfunction in an otherwise intact endothelium up to the rupture of a lipid-rich atherosclerotic plaque, leading to acute myocardial infarction or sudden death [17,27]. Antioxidants (e.g. albumin, bilirubin and glutathione) scavenge ROS, thus inhibiting the chain of reactions associated with endothelial dysfunction and atherogenesis [16].

2.4. Extracellular matrix components and their association with inflammatory markers

A few ECM components like vitronectin, fibronectin, laminin and type IV collagen play a role in ECM remodelling, modulate intracellular signalling and play a role in the remodelling of enzymes such as the MMPs [28]. Fibronectin is a collagenous glycoprotein produced by fibroblasts contributing to the structural framework of many cell surface receptor systems [18,29]. Laminins, on the other hand, are mainly presented in the basement membranes and are partly responsible for providing the tensile strength of the tissue [29,30]. Lastly, vitronectin is a glycoprotein that can bind to and regulate components of the plasminogen activator signal complex, in addition to its cell adhesion duties [29,31,32].

CVDs can be presented with an altered expression of plasma concentration of inflammatory markers and mediators, in particular a decrease in albumin [16,33] and an increase in C-reactive protein (CRP) concentration [34]. Albumin is a highly soluble protein present in human plasma at normal concentrations between 35 and 50 g/l [27]. The functions of albumin include the transport of metals, fatty acids, cholesterol, bile pigments and drugs [27]. Inflammation enhances vascular permeability mainly through chemicals released by activated damage [27]. The main factor affecting plasma albumin concentration in patients is the rate of transcapillary escape into the interstitial fluid [35]. This transcapillary escape of albumin is markedly increased in disease, leading to decreased plasma albumin concentration [35]. It is found that

postoperative patients and patients with severe infection will have low plasma albumin concentrations [35].

CRP is synthesized by the liver and regulated by interleukin-6 (IL-6) [36]. CRP is associated with the severity of atherosclerosis [36]. Elevated IL-6 levels were reported in diseases where inflammatory processes may facilitate the transition from clinically stable to unstable atherosclerotic plaque [36]. These observations imply that atheroma progression, as well as plaque rupture, may be predicted by raised CRP levels [36]. CRP induces the production of inflammatory cytokines in monocytes, promotes tissue factor expression in monocytes, is chemotactic for monocytes and induces shedding cell adhesion molecules [24]. It is also present in the foam cells in atherosclerotic lesions and co-localizes with activated fragments of the complement system [24]. A study demonstrated that people with high CRP levels had impaired endothelial vasoreactivity [24]. CRP stimulates MMP-1 expression by means of extracellular signal-related kinase pathway suggesting that CRP may promote matrix degradation and thus contribute to plaque vulnerability [37-39] .

2.5. Soluble urokinase-type plasminogen activator receptor (suPAR) and fibulin-1 as potential mediators in the development of sclerotic diseases

Against the background depicted in the previous section, this section focuses on two novel biomarkers namely fibulin-1 and suPAR in an attempt to elucidate their shared role in both the ECM and inflammatory system with emphasis on sclerotic CVD states.

2.5.1. Soluble urokinase-type Plasminogen Activator Receptor (suPAR)

2.5.1.1. The urokinase plasminogen activator system

The urokinase-type plasminogen activator (uPA) system consists mainly of the serine protease uPA, its cell membrane-associated receptor (uPAR), the soluble membrane-associated receptor (suPAR), a substrate called plasminogen and the plasminogen activator inhibitors (PAI) namely PAI-1 and PAI-2 [40,41]. The fibrinolytic system is responsible for converting plasminogen to an

active enzyme, plasmin. UPA, being an important component in this process, is produced by vascular endothelial cells, smooth muscle cells (SMC), monocytes, macrophages, fibroblasts, epithelial cells and also by malignant tumour cells of different origin [40,42]. UPA binds with high affinity to its receptor uPAR on the cell surface [40]. The main roles of the uPA system include cell migration, differentiation, proliferation and matrix degradation [40,43].

2.5.1.2. Urokinase-type Plasminogen Activator Receptor (uPAR)

UPAR is bound to the membrane by a glycosyl phosphatidyl inositol (GPI) anchor, having a single chain polypeptide that binds both single-chain pro-uPA and the active two-chain uPA [40]. Receptor-bound uPA activated plasminogen are a multifunctional receptor that promotes pericellular proteolysis, interacts with integrins such as vitronectin and thus facilitates cell-matrix intracellular signalling pathways [40,44-47]. uPAR can regulate monocyte adhesion by direct binding to vitronectin and by forming complexes with integrins [40].

2.5.1.3. SuPAR

A soluble bioactive form of uPAR, suPAR, is shedded or cleaved from the cell surface to body fluids, including plasma and urine [40]. SuPAR is secreted by inflammatory cells such as monocytic cells, macrophages and endothelial cells at a concentration of 0.8 – 3 ng/ml, in the plasma, urine and serum [49]. It consists of three homologue domains (DI, DII and DII) that form three associations namely the DI-DII association, the DII-DIII association and the DIII-DI association that are formed by means of three hydrogen bonds between the two domains [50]. The region connecting DI and DII-DIII can be cleaved by several different proteases including the uPAR ligand (uPA), plasmin, chymotrypsin, various MMPs and elastases [49,51,52].

Cell migration and cell adhesion play major roles in extracellular matrix degradation, a profound function of suPAR in disease states such as infection, autoimmune diseases, cancer and in cardiovascular disease including atherosclerosis [49,53,54]. Eugen-Olsen et al. found that participants with the highest suPAR levels have increased mortality in terms of cardiovascular outcome [53].

2.5.2. Fibulin

Fibulins are a family of extracellular matrix and blood glycoproteins consisting of seven members [55-59]. Approximately 25 years ago, Argraves et al. found that fibulins can interact with the cytoplasmic domain of the integrin β_1 subunit and the $\alpha_5\beta_1$ fibronectin receptor [55,60]. The fibulins become incorporated into the extracellular matrix similar to that of fibronectin [61]. Therefore, it is believed to function as intramolecular bridges that stabilise the organisation of supramolecular ECM such as elastic fibers and basement membranes [61].

2.5.2.1. Fibulin-1

In adults, fibulin-1 is expressed in the cardiac septa, valves, great vessels and is localised in skin and blood vessel walls [58,62]. Fibulin-1 is also found in the basement membranes where it contributes to the supramolecular organisation of ECM architecture [63]. In the stroma of most tissues, the extracellular matrix and blood plasma fibulin-1 is normally expressed at a concentration of 10–50 μg/ml [55-58,64-66]. Fibulin-1 also interacts with a variety of extracellular ligands in vitro, including elastin, endostatin, fibrinogen, integrins, proteoglycans and various basement membrane components [66-72].

The biochemistry of fibulin-1 contains an additional protein domain (domain I) at the N-terminus, which consists of three anaphylatoxin-like motifs [73]. It possesses two unique features not shared by the other family members. Its domain III is variable because of alternative splicing [55,56] and the other feature is that fibulin-1 interacts with fibrinogen and might be involved in haemostasis and thrombosis in the ECM and blood plasma [55,57,66].

Fibulin-1 plays a role in cell adhesion and migration along protein fibres within the ECM and is important for certain developmental processes [63]. It also plays a role in haemostasis and thrombosis owing to its ability to bind fibrinogen and incorporate into clots [74]. Fibulin-1 contributes to the structural integrity of the cardiac connective tissues [73] and may be associated with a subset of elastin-containing fibres [58]. The functional significance of fibulin-1

as an elastic fibre core possibility is that fibulin-1 serves a structural role, perhaps acting to stabilize the fibres through lateral interactions with elastin molecules or with components of microfibrils that both coat elastic fibres and penetrate into their cores [58]. The second possibility is that fibulin-1 plays a role in elastic fibrogenesis [58]. Fibulin-1 inhibits cell-adhesion and migration on fibronectin although binding of fibulin-1 does not block the integrin-binding sites of fibronectin, which led to the proposal that the formation of this complex generates a new anti-adhesive site, which repulses cellular interactions rather than promotes them [61,75].

2.6. The possible cross-link between fibulin-1 and suPAR in ECM degradation

Fibulin-1 and suPAR have separate functions, but it is possible that a connection exists by means of their collective purpose in ECM haemostasis. If the haemodynamic of the vascular system change, for example elevated blood pressure and flow, then shear stress on the vascular endothelium will increase [76]. In response to the stress, the production of ROS such as superoxide and hydrogen peroxide are stimulated as well as inflammatory cytokines such as IL-6 [11,19-21].

Similar to CRP, suPAR was found to be released in response to IL-6 secretion [36,53]. These cytokines cause the neutrophils to invade an inflamed area from the blood and initiate an increased expression of ICAM-1 on the surface of endothelial cells in the capillaries and venules [11]. Activation of the fibrinolytic system occurs in response to the conversion of plasma protein plasminogen to plasmin, which degrades fibrin and cause extracellular matrix degradation [40]. Fibrinogen and fibrin are degradation products of stable and unstable atherosclerotic plaques as well as a result of macrophages accumulating in sites of tissue damage, mediating endocytosis of both fibrinogen and fibrin monomers [78]. Fibulin-1 is incorporated into fibrin clots and binds to lipoprotein-(a) and mediates the accumulation of atherogenic lesions and may regulate thrombus formation at these sites [55]. The fibulin-1-fibrinogen-fibulin-1-fibrin complex could permeate from the blood into the intimal layer perhaps due to endothelial leakage [55]. Roark et al. proposed that fibulin-1 serves a structural role, possibly acting to stabilise the fibres through lateral interactions with elastin molecules or with components of microfibrils that both coat

elastic fibres and penetrate into their cores and may play a role in elastic fibre constituents in the formation of elastic fibres [58].

In the event where the elastin fibres within the intima-media are degraded and the collagens increase, arterial compliance decreases and contributes to the onset and progression of arterial stiffness [79]. Vitronectin is an adhesive extracellular matrix plasma protein found in blood vessel walls [80,81]. Studies have confirmed an interaction between fibulin-1 and fibrinogen as well as suPAR and vitronectin [80-82]. This may support the function of adhesion and has been implicated in several physiological and pathological processes [80-82].

2.7. Fibulin-1, suPAR and arterial stiffness

Arterial stiffness is the reduced capacity of arteries to dilate and contract optimally [83]. During changes in structural and functional properties, the arteries react by means of arterial remodelling to adapt to these changes [83]. In the event where the elastin fibres within the intima-media are degraded and the collagens increase, distensibility decreases and contributes to elevated arterial stiffness [21].

Fibulin-1 accumulates in the circulation and arterial walls of patients with type 2 diabetes, and appears to be a factor associated with arterial ECM changes in those patients [84]. Basal levels of pro-inflammation and oxidative stress are associated with arterial stiffness and hypertension by means of endothelial dysfunction [84]. A study showed an association between increased levels of fibulin-1 and arterial stiffness [85]. Although it is evident that Africans have a higher prevalence of arterial stiffness [2], studies on the association between fibulin-1 and arterial stiffness within this study population had not previously been investigated.

suPAR on the other hand is involved in the inflammatory process, which eventually may lead to arterial stiffness through atherosclerosis and subsequent turnover to arteriosclerosis [80-82]. None of the cross-sectional studies done on a bi-ethnic South African population have found an

association between suPAR and arterial stiffness [1,77], despite the strong association of suPAR with atherosclerotic plaque done in other studies [80-82,86].

2.8. SuPAR and fibulin-1, as a marker of cardiac fibrosis

Fibrosis occurs when ECM synthesis outpaces degradation [80-82]. Fibroblasts have the ability to secrete and break down proteins that form the ECM [87]. Cardiac fibroblasts maintain the ECM homeostasis which includes collagen, proteoglycans, glycoproteins, cytokines, growth factors and proteases [88,89]. During CVDs, distal regions of the heart typically undergo gradual reactive fibrotic processes as diffuse ECM synthesis proceeds [89,90]. Through the stimulation of fibroblast proliferation, increased pressure affects the ECM promoting cardiac fibrosis by stiffening and impairing contraction and relaxation of the myocardium [87].

The mechanism through which cardiac fibrosis develops can be explained by a few mediators. One such a mediator is galectin-3, demonstrated in different human fibrotic conditions [87,91]. Macrophage secretion and galectin-3 expression are major mechanisms in myofibroblast accumulation and activation and subsequent cardiac fibrosis [92,93]. Circulating plasma concentrations of B-type natruiretic peptide (BNP) and N-terminal pro-BNP (NT-proBNP) are currently the most commonly used biomarkers in heart failure and their levels are generally increased in proportion to the severity of the myocardial stretch or overload [92]. NT-proBNP was also found to associate with fibulin-1 that may contribute to cardiac alterations [92,93]. This may link fibulin-1 as a marker of cardiac fibrosis. Another study suggested that macrophage accumulation and increased plasminogen activator activity contribute to cardiac fibrosis [94]. Since suPAR is part of the plasminogen system, suPAR can also be expected to play a part in this process; however, further studies are required to confirm this statement.

2.9. Demographic and related disease perspectives of the South African population

Non-communicable diseases share common risk factors such as urbanisation and unhealthy diet that translate into CVD, diabetes and cancer [95]. The burden of non-communicable diseases is rising in low and middle income countries and rural communities [96]. The World

Health Organisation (WHO) found that the burden from non-communicable disease is two to three times higher in South Africa than in developed countries [99,100].

The South African population consists of a diversity of ethnic and cultural groups with different traditional eating patterns [96,101]. The African population in rural areas follows a very traditional diet whereas the African and Caucasian populations in urbanised areas consume a typical Western diet, possibly due to the socio-economic difference [102]. The traditional diet is associated with a low prevalence of degenerative diseases such as fibrosis and sclerosis, whereas the Western diet is associated with an increased prevalence of these diseases [102,103].

Studies have indicated that Africans are subjected to early changes within the vasculature that elevates their risk for the development of CVD due to the previously mentioned risk factors [103]. Possible markers that could contribute to the development of CVD can be important in combating the increasing risk of CVD in developing countries. A few of those markers such as suPAR, CRP, albumin and fibulin-1 have partly been investigated in European and American countries [1,77,104]. The marker fibulin-1 with other inflammatory markers such as suPAR, CRP and albumin in the inflammatory process can possibly provide a link between heart disease and inflammation, providing some answers to the role they play in cardiac fibrosis and ECM remodelling in developing countries, like South Africa, by preventing further increases in CVD.

2.10. Aims and hypotheses

Based on the available literature, the following aims and hypotheses were formulated:

Aims

Our study aims are to:

- Compare the levels of fibulin-1 and suPAR, CRP and albumin between African and Caucasian men and women; and to
- Explore whether fibulin-1 is independently associated with the inflammatory markers suPAR, CRP and albumin in a bi-ethnic cohort from South Africa.

Hypotheses

The following hypotheses were formulated:

- Fibulin-1, suPAR and CRP levels are higher and albumin levels lower in Africans.
- Fibulin-1 associates positively with suPAR and CRP and negatively with albumin in African and Caucasian men and women.

2.11. References

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Chapter 3

Manuscript

Instructions for authors: Atherosclerosis

The types of papers that can be submitted for consideration by the Editorial Board of *Atherosclerosis* include basic research papers that report results of original research or investigation using in vitro cell culture or animal models as well as clinical research papers reporting results of original clinical research or investigation in human subjects. The papers should not exceed 4000 words (including legends to figures and tables). No more than 5 figures and tables in total (e.g. 1 figure consisting of panels A and B and 4 tables). Authors are encouraged to include additional figures and tables as supplementary appendixes.

Manuscripts

Original articles should report original research not previously published or being considered for publication elsewhere. Manuscripts should be written in the English language (using either American or British spelling). As a rule, research papers should be divided into sections, headed by a caption (e.g. Abstract, Introduction, Materials, Methods, Experimental Results, Discussion, etc.). Please include a short paragraph of conclusions (at the end of the text), indicating the relevance of the study with regard to the basics and/or clinical aspect of atherosclerosis. A statement concerning the source of funding, conflicts of interests and disclosures of financial support is highly recommended.

→ Essential title page information

- *Title*: Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- *Author names and affiliations*: Where the family name may be ambiguous (e.g. a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

- *Corresponding author*: Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.
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A structured abstract (objective, methods, results and conclusion) of 50-250 words must be included.

→ Keywords

A keyword summary must be provided; normally 3-7 items should be included. Authors are encouraged to choose their own keywords but, if in grave doubt which items to select, *Medical Subject Headings* (issued with the January *Index Medicus, 1969)* may be used as a guideline.

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Tables with titles and legends must be on separate pages with double spacing; they may be included in the same file as the manuscript text or in separate file(s). Authors must list on the title page or in the covering e-mail, the number of figures and/or tables to be found in the paper.

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There are no strict requirements on reference formatting. References can be in any style or format as long as the style is consistent. Author(s) name(s), journal title/book title, article title, year of publication, volume number/book chapter number and the pagination must be present. The reference style required by the journal will be applied to the published version by Elsevier.

Exploring a marker of cardiac fibrosis and its association with soluble uPAR in a bi-ethnic South African population: The SAfrEIC study

CS du Plooy,[a] HW Huisman[a],* R Kruger,[a] AE Schutte[a].

[a]Hypertension in Africa Research Team (HART), North-West University, Potchefstroom, South Africa

Number of figures: 2

Number of tables: 3

*Corresponding author:

Prof. HW Huisman

Hypertension in Africa Research Team (HART)

North-West University (Potchefstroom Campus)

Private bag x6001

Potchefstroom

2520

South Africa

Email: hugo.huisman@nwu.ac.za

Tel: +27 18 299 2439

Fax: +27 18 299 1053

Abstract

Objectives: Two biomarkers emerged as mediators in the development of sclerotic disease, namely fibulin-1 and soluble urokinase-type plasminogen activator receptor (suPAR). The latter, along with C-reactive protein (CRP) and albumin, are known to delineate inflammatory processes associated with atherosclerosis. We aimed to explore the independent relationship of fibulin-1 with these inflammatory markers in a bi-ethnic South African population.

Methods: This study included 290 Africans (men: n=130 and women: n=160) and 343 Caucasians (men: n=160 and women: n=183). Serum fibulin-1, suPAR, CRP and albumin levels were measured along with conventional cardiovascular and metabolic variables.

Results: In both single and partial regression analyses (adjusting for age), fibulin-1 correlated with both suPAR and albumin in African men and with suPAR in Caucasian men. Fibulin-1 was not associated with suPAR and other inflammatory markers in women. In multivariate regression analysis the associations of fibulin-1 were confirmed in men (African: suPAR (R^2=0.22; β=0.329; p<0.001) and albumin (R^2=0.22; β=–0.210; p=0.013); Caucasian: suPAR (R^2=0.14; β=0.234; p=0.008)).

Conclusions: Our study found an independent positive association between fibulin-1 and suPAR in both African and Caucasian men. Fibulin-1 was also associated negatively with albumin in African men only. These results are indicative of the presence of potential subclinical inflammation as depicted by suPAR within the extracellular matrix of endothelial tissue, contributing to the potential onset of cardiac fibrosis or vascular sclerosis among these South African men with lower albumin levels.

Keywords: fibulin-1, suPAR, cardiac fibrosis, inflammation, extracellular matrix remodelling, African, Caucasian

Introduction

The cardiovascular extracellular matrix (ECM) scaffold undergoes continuous progressive and regressive changes; however, these changes differ between normal and pathological conditions. In pathological conditions such as hypertension, coronary artery disease and aortic stenosis, alterations in the ECM largely contribute to sclerotic processes [1]. Sclerotic progression includes the hardening, thickening or loss of resilience within connective tissue of organs such as the myocardium or the lining (intima-media) of blood vessel walls [2,3]. In disease states such as hypertension, hyperlipidemia or low-grade inflammation, multiple changes may occur over time and ultimately escalate into the development of atherosclerotic plaque and sclerotic lesions [4-7]. Two biomarkers, fibulin-1 [8-11] and soluble urokinase-type plasminogen activator receptor (suPAR), [12-14] emerged as potential mediators in the development and progression of fibrotic and sclerotic processes.

Fibulin-1 is a fibrinogen binding glycoprotein and is expressed in the cardiac septa, cardiac valves, the skin and blood vessel walls [10-13,17,18]. A few studies have partially explored the role of fibulin-1 in the cardiovascular system [15-18]. The most conspicuous functions of fibulin-1 include cell adhesion, cell migration within the ECM and the organisation of ECM architecture in especially the basement membrane of the vasculature [9,19]. Fibulin-1 may reflect vascular dysfunction and fibrosis in the myocardium as a result of inflammation, by means of changes in the ECM [17,20,21].

Inflammatory mediators (such as CRP, as well as the anti-inflammatory defence system consisting of endogenous antioxidants (such as albumin)) are implicated in inflammatory processes involved in endothelial dysfunction and the development of atherosclerosis [22,23]. SuPAR, a subclinical marker of inflammation-related atherosclerosis [13,14], is the soluble form of the urokinase-type plasminogen activator receptor (uPAR), a glycoprotein [12-14], released from inflammatory cells [24]. Fibulin-1 and suPAR function via different pathways, but are potentially connected in ECM remodelling related to arterial stiffness and cardiac fibrosis.

In South Africa, a few studies have found that Africans are subject to early changes within the vasculature such as stiffening and thickening of the blood vessel walls [15,25-27]. These studies also indicated that Africans are at higher risk for developing early cardiovascular disease due to lifestyle, an increased low-grade inflammatory profile and intrinsic risk factors [25-29]. Our study aims to compare the levels of fibulin-1, suPAR, CRP and albumin in African and Caucasian men and women. Also, in order to understand the potential functions of these markers we further aim to explore fibulin-1 and its potential association with suPAR, CRP and albumin.

Materials and methods

Study population

The cross-sectional SAfrEIC study (South African study regarding the role of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function) included an initial total of 756 African and Caucasian men and women from the North West Province in South Africa. The Ethics Committee of the North-West University, Potchefstroom Campus, approved the study. For the investigation of human subjects, the study protocol conforms to the ethical guidelines of the Declaration of Helsinki (as revised in 2008). HIV-infected participants (n=115) as well those with missing data (n=8) were excluded from this study. A total of 290 Africans (men: n=130; women: n=160) and 343 Caucasians (men: n=160; women: n=183) were included in this study.

Study protocol

Daily, 10 to 20 participants visited the Metabolic Unit facility at the North-West University on the Potchefstroom Campus. Participants were informed of all procedures and gave written consent. During the morning the participants completed a Basic Health and Demographic Questionnaire. In the event that abnormalities were identified after screening procedures, they were referred to their local clinic, hospital or physician.

Cardiovascular measurements

The Omron HEM-757 (Omron Healthcare, Kyoto, Japan) apparatus was used to determine systolic (SBP) and diastolic blood pressure (DBP) with the cuff on the left upper arm, whilst being in the sitting position. Each participant rested for 10 minutes prior to the blood pressure measurements after which recordings were done in duplicate at five minute intervals. Participants with a SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg were considered hypertensive [30]. The Finometer device (FMS, Finapres Medical Systems, Amsterdam, Netherlands) was used to determine total peripheral resistance and Windkessel arterial compliance. The Finometer was connected to the left arm and left index finger of the participant and measurements were recorded continuously for at least seven minutes [31]. The means of the cardiovascular variables of the last two minutes of the recordings were used for analyses.

Anthropometric measurements

The body height, body mass and waist circumference of each participant were measured in triplicate according to standard procedures [32]. The body height was measured to the nearest 1.0 cm using the Invicta stadiometer (Invicta Plastics 1465, Leicester, U.K) and body mass to the nearest 0.1 kg (Precision Health Scale, A & D Company, Japan). The waist circumference was measured with a Holtain non-stretchable metal flexible measuring tape. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared and rounded to 1 decimal point.

Blood sampling and biochemical analyses

All participants were requested to fast for at least 8 hours. A registered nurse acquired a fasting blood sample from the ante-brachial vein using a winged infusion set and measured the blood glucose level at the Metabolic Unit using enzymatic method to screen for diabetes mellitus (Life ScanSure Step® Blood Glucose Monitoring System, Life Scan Inc, Milputas, CA 9535). Serum samples were frozen at -80 °C prior to all further analyses. Fasting lipids (triglycerides), serum glucose, γ-glutamyl transferase (γ-GT), albumin, creatinine clearance and high sensitivity CRP

were determined with the KonelabTM 20i autoanalyser (Thermo Fisher Scientific Oy, Vantaa, Finland). Low-density lipoproteins were determined by the Friedewald formula [33]. EDTA-coagulated plasma was used to determine suPAR levels with the suPARnostic® enzyme-linked immunosorbant assay (ELISA) kit (ViroGates, Copenhagen, Denmark) and fibulin-1 by using a sandwich immunoassay as previously described [17]. Creatinine clearance was calculated by using Cockcroft-Gault equation [34]. Serum cotinine was determined with the IMMULITE 2000 nicotine metabolite assay (Siemans Medical Solutions Diagnostic Ltd., Los Angeles, CA).

Statistical analyses

Statistical analyses were performed using Statistica version 11 (Statsoft Inc. Tulsa, OK, 2012). Main effects of ethnicity and gender were tested on the associations between fibulin-1 and suPAR by means of multiple regression analyses. Variables that deviated from normality (cotinine, γ-GT, CRP) were logarithmically transformed prior to any further statistical analyses. T-test and Chi-square tests were used to compare means and proportions between African and Caucasian men and women. Single regression analyses were done to determine the relationships between fibulin-1, suPAR, CRP and albumin. Graphpad v5.03 (GraphPad Software, Inc., San Diego, California, USA) was used to plot the unadjusted correlations of fibulin-1 with suPAR, CRP and albumin between African and Caucasian men and women. Partial correlations were performed to determine the relationship of fibulin-1 with suPAR, CRP and albumin with adjustments for age. We divided suPAR, CRP and albumin values into tertiles to explore associations with fibulin-1 levels, while adjusting for age, BMI and DBP in analysis of covariance (ANCOVA). Independent associations were determined by performing forward stepwise regression analyses in the African and Caucasian men only. Variables considered for entry in the model were age, BMI, DBP, γ-GT, creatinine clearance, CRP, cotinine, suPAR and albumin. P-values of <0.05 were considered statistically significant.

Results

Basic descriptive characteristics of this study population are listed in Table 1. The participants were divided into African and Caucasian men and women due to significant interactions of

ethnicity and gender on the associations of fibulin-1 with suPAR (ethnicity: $F(633)=7.29$; $p<0.001$ and gender: $F(633)=10.12$; $p<0.001$). Additional interaction terms were introduced to test the main effects of inflammation on the association between fibulin-1 and CRP and fibulin-1 and albumin for both genders (CRP: $F(633)=6.26$; $p<0.001$ and albumin: $F(633)=8.33$; $p<0.001$) and ethnicity (CRP: $F(633)=4.12$; $p<0.05$ and albumin: $F(633)=5.69$; $p<0.001$)).

BMI and waist circumference were lower in African compared to Caucasian men ($p<0.001$), with no significant difference between African and Caucasian women ($p=0.78$ and $p=0.59$, respectively). In both gender groups suPAR levels were higher and albumin were lower in Africans compared to Caucasians ($p<0.006$). Fibulin-1 levels were higher in African men ($p=0.010$), whereas CRP was higher in African women ($p<0.001$) compared to their Caucasian counterparts. Low density lipoprotein cholesterol was higher in the Caucasian men and women (both $p<0.001$). Creatinine clearance was significantly lower in African than Caucasian men ($p<0.001$), with no significant difference in women. Africans had significantly higher SBP, DBP, total peripheral resistance and lower arterial compliance in comparison to Caucasians (all $p<0.05$) in both gender groups. The cotinine and γ-GT levels were also higher in the Africans compared to Caucasians (all $p<0.001$).

In single regression analysis, we found a positive correlation between fibulin-1 and suPAR in African ($r=0.19$; $p=0.028$) and Caucasian ($r=0.37$; $p<0.001$) men, also in African ($r=0.193$; $p=0.028$) and Caucasian women ($r=0.14$; $p=0.036$) (Figure 1). A negative correlation existed between fibulin-1 and albumin in African men ($r=–0.26$; $p=0.003$), with borderline significance in Caucasian men ($p=0.057$). No significant association was found between fibulin-1 and CRP in any of the groups. Partial correlations (adjusted for age) of fibulin-1 with suPAR, CRP and albumin are shown in Table 2. After adjustments, the positive correlation remained between fibulin-1 and suPAR in African ($r=0.23$; $p=0.010$) and Caucasian ($r=0.22$; $p=0.005$) men, but significance was lost in African and Caucasian women. An inverse correlation of fibulin-1 with albumin remained in African men ($r=–0.28$; $p=0.002$). No significant correlation existed between fibulin-1 and CRP in any of the groups. We plotted fibulin-1 levels by tertiles of suPAR, CRP and

albumin in both African and Caucasian men and women while adjusting for age, BMI and DBP (Figure 2). We used previously established categories to define CRP concentrations (low: <1mg/L; intermediate: 1-3 mg/L or high:>3mg/L) [35]. A positive trend remained between fibulin-1 and suPAR in both African and Caucasian men (p for trend =0.039 and p for trend =0.017, respectively). Fibulin-1 inversely correlated with albumin in African men only (p for trend <0.001).

Since no significant correlations existed in the African and Caucasian women, the forward stepwise multiple regression analysis was only performed in men (Table 3). After adjustment for covariates (age, BMI, DBP, γ-GT, CRP, albumin, creatinine clearance, cotinine and medication) the associations of fibulin-1 with suPAR (Adj. R^2=0.217; β=0.329; p< 0.001) and albumin (Adj. R^2=0.217; β=–0.210; p=0.013) persisted in African men. The association between fibulin-1 and suPAR also remained significant in Caucasian men (Adj. R^2=0.113; β=0.234; p=0.008).

Table 1: Descriptive characteristics of a bi-ethnic South African population

	Men n = 290			Women n = 343		
	African n = 130	**Caucasian n = 160**	**p-value**	**African n = 160**	**Caucasian n = 183**	**p-value**
Age (years)	41.2 ± 13.6	40.0 ± 13.0	0.43	42.2 ± 12.2	40.9 ± 12.9	0.32
Weight (kg)	58.2 ± 12.8	91.8 ± 17.4	< 0.001	68.4 ± 19.3	75.3 ± 17.7	< 0.001
Body mass index (kg/m^2)	20.4 ± 4.08	28.3 ± 4.96	< 0.001	27.6 ± 7.57	27.4 ± 6.40	0.78
Waist circumference (cm)	74.2 ± 10.2	94.5 ± 13.5	< 0.001	83.1 ± 14.3	82.3 ± 13.8	0.59
Biochemical analyses						
Fibulin-1 (µg/mL)	71.6 ± 21.3	65.7 ± 16.9	0.010	76.0 ± 24.0	72.1 ± 18.0	0.087
suPAR (ng/mL)	3.39 ± 2.26	2.24 ± 0.69	< 0.001	3.33 ± 1.37	2.36 ± 0.69	< 0.001
C-reactive protein (mg/L)	1.58 (1.09 – 2.29)	1.21 (0.93 – 1.58)	0.23	3.58 (2.81 – 4.56)	1.48 (1.15 – 1.89)	< 0.001
Albumin (g/L)	44.9 ± 7.24	48.3 ± 8.26	< 0.001	43.3 ± 4.62	45.4 ± 7.80	0.006
Creatinine clearance (mL/min)	111.6 ± 31.8	158.6 ± 39.6	< 0.001	105.9 ± 35.9	108.8 ± 32.6	0.46
Serum glucose (mmol/L)	5.03 ± 0.76	5.72 ± 0.81	< 0.001	5.34 ± 1.37	5.42 ± 1.40	0.60
Low density lipoprotein (mmol/L)	2.24 ± 0.88	3.82 ± 1.25	< 0.001	2.53 ± 0.83	3.73 ± 1.21	< 0.001
High density lipoprotein (mmol/L)	1.68 ± 0.77	1.19 ± 0.36	< 0.001	1.44 ± 0.46	1.54 ± 0.38	0.036
Triglycerides (mmol/L)	1.11 ± 0.58	1.75 ± 1.37	< 0.001	1.25 ± 0.84	1.37 ± 0.76	0.18
Cardiovascular measurements						
Systolic blood pressure (mmHg)	130.1 ± 20.8	124.9 ± 13.1	0.012	123.4 ± 22.5	114.9 ± 16.4	< 0.001
Diastolic blood pressure (mmHg)	84.2 ± 13.9	79.7 ± 9.07	< 0.001	85.1 ± 13.5	77.0 ± 10.3	< 0.001
Total peripheral resistance (mmHg/L/min)	1.41 ± 0.63	1.00 ± 0.31	< 0.001	1.36 ± 0.52	1.10 ± 0.33	< 0.001
Arterial compliance (mL/mmHg)	1.68 ± 0.49	2.40 ± 0.60	< 0.001	1.54 ± 0.49	1.88 ± 0.44	< 0.001
Lifestyle						
Hypertension status, *n* (%)	50 (38.5)	24 (15.0)	< 0.001	46 (34.8)	26 (12.3)	< 0.001
Cotinine (ng/mL)	125.9 (97.0 – 163.6)	18.3 (14.9 – 22.5)	< 0.001	63.2 (47.4 – 84.4)	12.0 (10.3 – 14.1)	< 0.001
γ-glutamyl transferase (U/L)	79.3 (66.8 – 94.1)	37.9 (34.9 – 41.3)	< 0.001	54.6 (46.1 – 64.6)	24.2 (22.2 – 26.3)	< 0.001
Co-morbidities, *n* (%)*	0 (0)	47 (29.4)	< 0.001	2 (1.52)	54 (25.6)	< 0.001

Values are arithmetic mean ± SD, geometric mean (5th and 95th confidence interval) or number of participants.
*refers collectively to anti-inflammatory, anti-diabetic and anti-hypertensive medication. Abbreviations: HOMA: Homeostasis model assessment; suPAR: soluble urokinase-type plasminogen activator receptor.

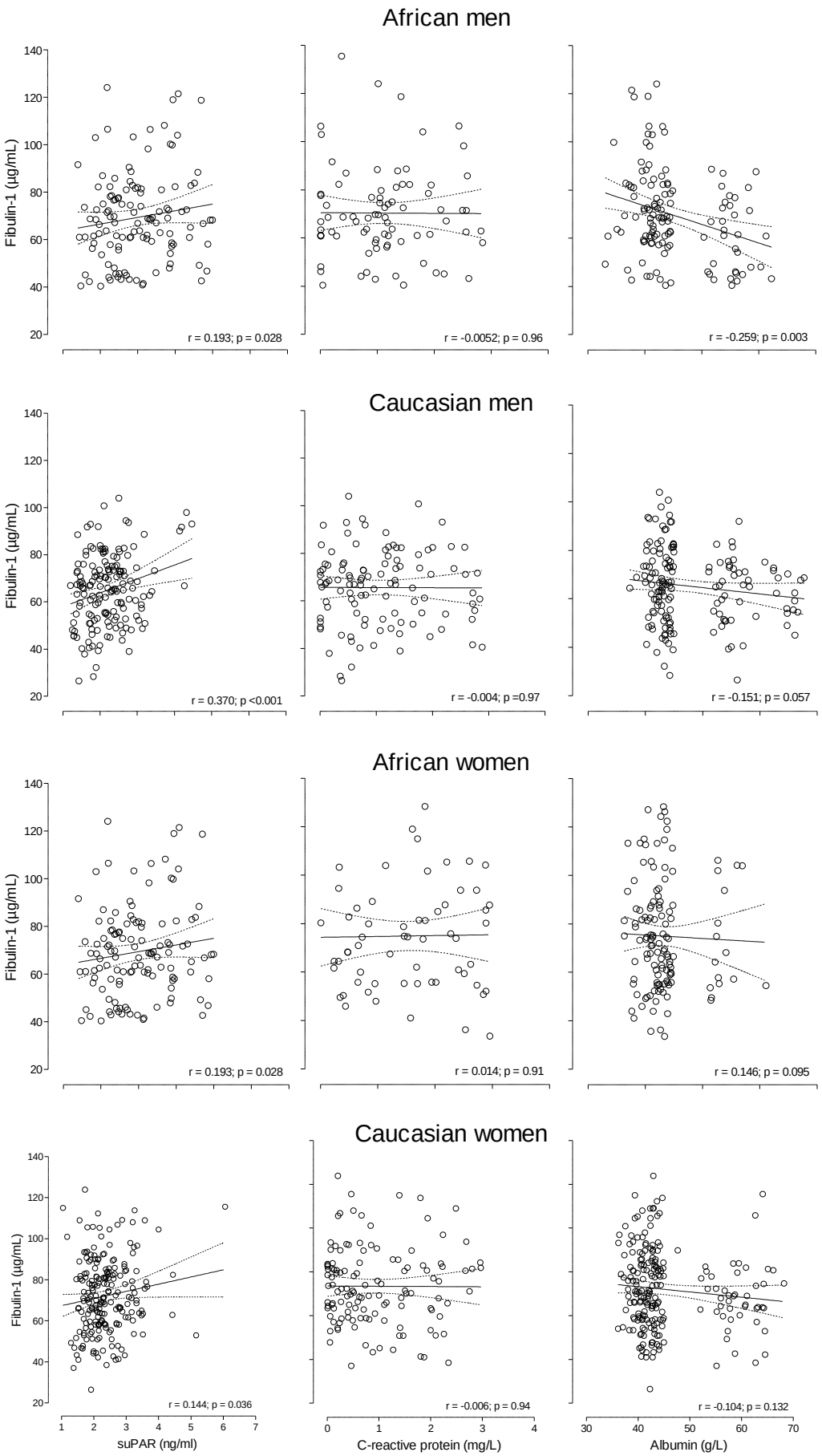

Figure 1 : Fibulin-1 with suPAR, CRP and albumin in African and Caucasian men and women. Abbreviations: suPAR: soluble urokinase-type Plasminogen Activator Receptor.

Table 2: Partial correlations of fibulin-1 with suPAR, CRP and albumin in African and Caucasian men and women

	Men **n = 290**		**Women** **n = 343**	
	African **n = 130**	**Caucasian** **n = 160**	**African** **n = 160**	**Caucasian** **n = 183**
suPAR (ng/mL)	r = 0.23; p = 0.010	r = 0.22; p = 0.005	r = –0.04; p = 0.68	r = 0.09; p = 0.18
CRP (mg/L)	r = 0.08; p = 0.35	r = –0.03; p = 0.75	r = 0.13; p = 0.13	r = –0.06; p = 0.13
Albumin (g/L)	r = -0.28; p = 0.002	r = -0.09; p = 0.245	r = 0.13; p = 0.14	r = –0.10; p = 0.13

Adjustments applied for age. Abbreviations: suPAR: soluble urokinase-type Plasminogen Activator Receptor; CRP: C-reactive protein

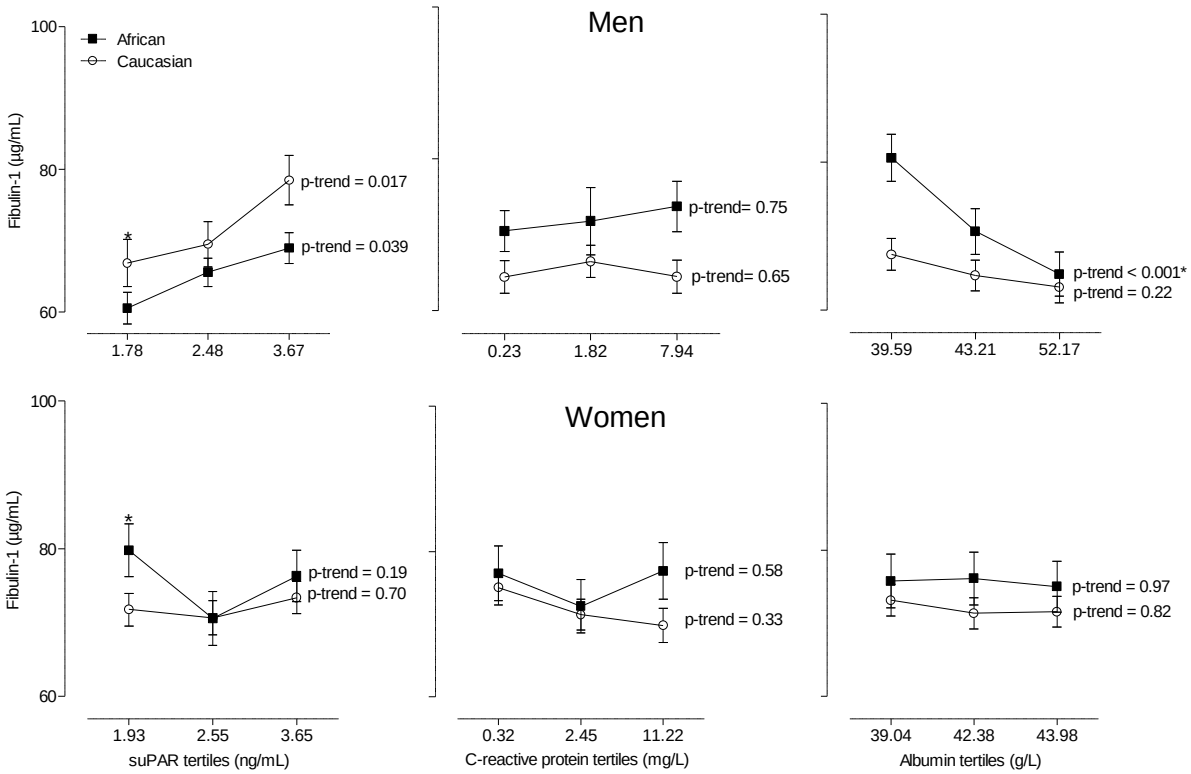

Figure 2: Tertiles of fibulin-1 with suPAR, CRP and albumin against fibulin-1 (adjusted for age, BMI and diastolic blood pressure). *p<0.05 ($tertile_{African}$ vs. $tertile_{Caucasian}$), † p<0.05 ($tertile_1$ vs. $tertile_3$). Abbreviations: suPAR: soluble urokinase-type Plasminogen Activator Receptor.

Table 3: Forward stepwise regression analysis of fibulin-1 as dependent variable with suPAR in African and Caucasian men

	Fibulin-1 (µg/ml)			
	African men		**Caucasian men**	
	n = 130		**n = 160**	
Adjusted R^2	0.217		0.113	
	Std β (95% CI)	p-value	Std β (95% CI)	p-value
suPAR (ng/mL)	0.329 (0.147 to 0.513)	< 0.001	0.234 (0.063 to 0.404)	0.008
Albumin (g/L)	–0.210 (–0.373 to –0.046)	0.013	–	–
Diastolic blood pressure (mm Hg)	–0.209 (–0.375 to –0.044)	0.015	–	–
γ-glutamyl transferase (U/L)	–0.221 (–0.404 to –0.039)	0.019	–0.188 (–0.296 to 0.010)	0.018
Cotinine (ng/mL)	–0.255 (–0.418 to –0.093)	0.003	–0.131 (–0.317 to –0.001)	0.108
Medication for co-morbidities*	–	–	0.192 (0.026 to 0.359)	0.024

Variables that did not enter the model are indicated with –. CRP, estimated creatinine clearance, age and BMI did not enter the model for both African and Caucasian men. * refers collectively to anti-inflammatory, anti-diabetic and anti-hypertensive medication. Abbreviations: suPAR: soluble urokinase-type plasminogen activator receptor; Std β: standardized regression beta coefficients; CI: confidence interval.

Discussion

In this study we explored the independent association of fibulin-1 (a marker of cardiac fibrosis) with suPAR, CRP and albumin within a bi-ethnic South African population. We found a positive association between fibulin-1 and suPAR in African and Caucasian men, but not in women. In addition, an inverse relationship was found between plasma fibulin-1 and serum albumin levels in African men only. The stronger association of fibulin-1 with suPAR and negative association with albumin in African men suggest that early cardiovascular ECM alterations are present in African men, also reflected by higher fibulin-1 and suPAR concentrations in these men.

The absence of the abovementioned associations in women (after adjustment for age) may be explained by the role of female hormones in the age dependent stages of uterine and ovarian cycles. This study lacked the hormonal profile for each female participant. Nevertheless, gender has an important influence on the incidence of CVD, with men and postmenopausal women having a greater disease burden than reproductive age cycling women [36]. Estrogens play an important anti-inflammatory role during inflammation [36-38]. During increased oxidative stress, estrogens up-regulate superoxide dismutase or can detoxify the superoxide ions [37,38]. Estrogens have the capacity to reduce oxidative stress and inflammation through their antioxidant properties [36]. This suggests that in the female, inflammatory processes are implicated through a different physiological pathway than men, due to higher levels of estrogens. This might explain the absence of the association of fibulin-1 with suPAR, CRP and albumin in African and Caucasian women.

Previous studies have investigated to what extent increased circulating inflammatory markers (CRP and suPAR) contribute to the progression of atherosclerotic processes in vascular dysfunction [39-41]. Africans are subjected to early changes within the vasculature, increasing their risk for developing cardiovascular disease, especially observed in men [25,27,42-44]. To our knowledge, the potential links that may exist between fibulin-1 and suPAR in the development and progression of fibrotic and sclerotic processes has not been investigated yet.

Our study showed that fibulin-1 and suPAR levels are higher and more strongly associated in African compared to Caucasian men. This result suggests a link between the potential developments of subclinical damage in the form of early-onset atherosclerosis, especially in African men.

Intrinsic physiological components with antioxidant properties protect the vasculature from reactive oxygen species in the event of atherosclerosis development [45,46]. One such antioxidant is albumin, a protein present in inflammation as well as the aqueous core of extracellular lipoproteins of atherosclerotic vessels, which have protective effects against harmful oxidation [46]. Our study shows that with increasing levels of fibulin-1 in African men, albumin levels decline, and this is in line with a few other studies [22,23,47-50]. Albumin plasma concentration will decrease markedly in patients with diseases where inflammation occurs [22,23,47-50]. This finding may support previous reported early-onset vascular changes and increasing risk for developing cardiovascular disease in this population group.

CRP and suPAR release are regulated by interleukin-6 (IL-6) associated with the severity of atherosclerosis [51,52]. This leads to the activation of the fibrinolytic system which degrades fibrin and cause extracellular matrix degradation [39]. Fibulin-1 is incorporated into fibrin clots, binds to lipoprotein-(a) (LP(a)) and mediates the accumulation of atherogenic lesions and may regulate thrombus formation at these sites [53]. LP(a) concentrations have been shown to differ among ethnic groups, with Africans having LP(a) levels several times higher than Caucasians [54]. This may be another possible explanation to how fibulin-1 and suPAR can contribute to the early-onset vascular changes in the black population, although LP(a) data were not available in this study.

It is noteworthy to mention the significant independent negative associations of fibulin-1 with GGT and cotinine in our study. These inverse associations are unexpected due to the well-known detrimental cardiovascular disease effects of excessive alcohol use and smoking [55,56]. In fact, we found increased levels of GGT and cotinine in the Africans compared to the

Caucasians. The mechanisms by which these markers contribute to vascular disease are not yet completely understood, and we therefore encourage further studies.

The results of this study need to be interpreted within its context of limitations and strengths. This was a cross-sectional study and we cannot pinpoint any causes or effects. We did not assess plaque formation or presence by means of vascular ultrasound or invasive techniques. Conventional markers of ECM scaffold (i.e. elastin and collagens) were not measured. Although other methods of classifying and diagnosing cardiac fibrosis are known, we explored fibulin-1 as an alternative screening tool for fibrosis to aid as viable substitute in clinics that do not have the means for analysing advanced molecular biology as a standard protocol. However, this study was the first to explore the potential association between fibulin-1 and suPAR along with haemodynamic and metabolic components in a bi-ethnic South African population. The sample size of our groups was of valued size, but future longitudinal studies and larger epidemiologic studies are encouraged to confirm these findings.

In conclusion, our study confirms a persistent link between a surrogate marker of cardiac fibrosis (fibulin-1) and inflammation (as presented by suPAR) in African and Caucasian men. We also found an inverse relationship between fibulin-1 and vascular protective albumin in African men. Our results suggest that these South African men are subject to a decline in vascular protective antioxidants resulting in early development of vascular dysfunction and potential future subclinical vascular sclerosis or cardiac fibrosis.

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Chapter 4

General conclusions and recommendations

4.1. Introduction

In this chapter the main findings from this study are summarised. The results are discussed, interpreted, explained and compared to relevant literature. Conclusions are drawn and recommendations are made to researchers investigating fibulin-1, a marker of cardiac fibrosis, and soluble urokinase-type plasminogen activator receptor (suPAR), along with other markers of inflammation (CRP and albumin).

This study aimed to compare the levels of fibulin-1, suPAR, CRP and albumin in African and Caucasian men and women. It also aimed to explore fibulin-1 and its potential association with inflammatory markers suPAR, CRP and albumin in relation to other haemodynamic and metabolic components in a bi-ethnic cohort from South Africa.

4.2. Summary, discussion and comparison to relevant literature

Hypothesis 1*: Fibulin-1 and suPAR levels are higher and albumin levels are lower in Africans than their Caucasian counterparts.*

The results of this study indicated that fibulin-1 and suPAR levels were higher in Africans compared to Caucasians in both gender groups. This finding confirm previous studies showing higher levels of inflammatory markers in African compared to Caucasian populations[1,2]. Therefore the first hypothesis can be accepted. The higher fibulin-1 and suPAR levels of the African population may be driven by inflammation. The result also suggests the link between the potential developments of subclinical damage in coordination with early onset atherosclerosis. Besides previous publications by our group on the same sample population [3,4], fibulin-1 was not investigated in different ethnic groups before. Therefore comparison of findings with the literature is difficult.

***Hypothesis 2**: Fibulin-1 is positively associated with suPAR and CRP and negatively with albumin in African and Caucasian men and women.*

A forward stepwise multiple regression analysis confirmed the association between fibulin-1 and suPAR in African and Caucasian men, with an inverse relationship of fibulin-1 with albumin in African men only. Therefore the second hypothesis is partially accepted. We found no association between fibulin-1 and CRP in any of the groups. The results of fibulin-1 and the positive association with suPAR, CRP and negative association with albumin were absent in all women.

Cardiovascular diseases such as atherosclerosis and cardiac fibrosis have an undertone of extracellular matrix remodelling in the form of sclerosis [5]. Fibulin-1, a marker of cardiac fibrosis, and suPAR a marker of inflammation, along with other markers of inflammation (CRP and albumin), received some attention due to their contributions to matrix alteration [6-9]. A previous study proposed that suPAR is associated with cardiovascular disease; however, the study was performed on Caucasians only [10]. Our study was the first to investigate the potential associations between all these components.

In a previous study done on the association of CRP with other cardiovascular risk factors Folsem et al., found that CRP values were higher in women than in men, and higher in African-Americans than in whites [11]. This is supported by this study with the CRP values being higher in the African women compared to their Caucasian counterparts. The study mentioned also found that CRP was not associated with a marker of atherosclerosis (carotid intima-media thickness) [11], confirmed by this study that found no association of CRP with fibulin-1 (a possible link for the development and progression of fibrotic and sclerotic processes).

A few studies done on albumin that have suggested that albumin plasma concentration will decrease remarkably in patients with diseases where inflammation occurs [12-14], are supported by this study in African men that with decreasing levels of albumin the level of fibulin-

1 increase. Serum albumin was inversely associated with cardiovascular mortality in men and women, older and younger persons and African Americans and whites in several studies [15-17].

The stronger association of fibulin-1 with suPAR and additional inverse association with albumin in African men suggest that early cardiovascular ECM alterations are present in African men. After partial correlation and multiple regression analysis the association between fibulin-1 with suPAR and albumin in the men only, suggests that the female body deals with these inflammatory processes through a different physiological pathway [18-20]. Although the findings cannot reflect the status of the whole South African population, it could provide a reference for future studies.

4.3. Study limitations

There are possible aspects that may have affected the results of this study, thus it is important to reflect on some of the limitations.

This was a cross-sectional study, therefore conclusions about cause and effect or sequence of events cannot be made. Methodologically this study did not assess plaque formation or presence by means of vascular ultrasound or invasive techniques and neither were conventional markers of ECM scaffold measured, such as elastin and collagens.

The sample size of the group in this study is of valued size (African: men: n=130, women: n=160; Caucasian: men: n=160, women: n=183), but future longitudinal studies and larger epidemiologic studies are encouraged to confirm these findings. Results obtained from this study revealed the general health of an availability sample from the population, thus the entire South African population cannot be represented by this study group.

The findings of this study could have also been influenced by the differences in socio-economic background that could explain the differences in the findings of this study. The Africans

consisted mostly of poor and unemployed participants, whereas Caucasians were from a higher socio-economic class. However, double control cannot be applied. Since an interaction existed with ethnicity, additional adjustment for social-economic class would reveal no different result, unless the whole population was stratified in categories of monthly income (which was not the scope and focus of this study).

The possibility of chance should also be considered. By adjusting for confounders such as age, body mass index, diastolic blood pressure, gamma glutamyl transferase, creatinine clearance, CRP, cotinine, suPAR, albumin and medication for co-morbidities (anti-inflammatory, anti-diabetic and anti-hypertensive medication) through partial correlation or forward stepwise analyses, it is possible that these confounders may have influenced the results by causing an over- or underestimation of the associations between fibulin-1 with suPAR, CRP and albumin. Statistics indicated that by using partial correlation and forward stepwise regression analysis, significant correlations may be one out of twenty, due to chance.

4.4. Conclusion

In conclusion, our study confirms a persistent link between a surrogate marker of cardiac fibrosis (fibulin-1) and subclinical inflammation (as presented by suPAR) in African and Caucasian men. We also found an inverse relationship between fibulin-1 and vascular protective albumin in African men. Our results suggest that these South African men are subjected to a decline in vascular protective antioxidants resulting in early development of vascular dysfunction and potential future subclinical vascular sclerosis or cardiac fibrosis.

4.5. Future relevance

The following recommendations are proposed for future studies:

- Prospective studies and larger epidemiologic studies are encouraged to investigate a cause-effect relationship.
- Fibulin-1 and suPAR should be investigated amongst other population groups to determine whether global ethnic differences exist in fibulin-1 and suPAR.

- Plaque formation should be assessed to clarify whether fibulin-1 and suPAR are related to structural and functional changes within the ECM.
- Fibulin-1 and suPAR should be compared to conventional and also the latest golden standard markers for sclerotic and fibrotic disease states to validate their potential as surrogate markers in these disease states.

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