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CVD risk factors are related to plasma fibrin clot properties independent of total and or γ' fibrinogen concentration

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ABSTRACT

Introduction: Cardiovascular disease (CVD) risk factors are associated with total fibrinogen concentration and/or altered clot structure. It is however, unclear whether such associations with clot structure are ascribed to fibrinogen concentration or other independent mechanisms. We aimed to determine whether CVD risk factors associated with increased total and/or γ' fibrinogen concentration, were also associated with altered fibrin clot properties and secondly whether such associations were due to the fibrinogen concentration or through independent associations.

Materials and methods: In a plasma setting CVD risk factors (including total and γ' fibrinogen concentration) were cross-sectionally analysed in 2010 apparently healthy black South African participants. Kinetics of clot formation (lag time, slope and maximum absorbance) as well as clot lysis times were calculated from turbidity curves.

Results: Of the measured CVD risk factors age, metabolic syndrome, C-reactive protein (CRP), high density lipoprotein (HDL)-cholesterol and homocysteine were significantly associated with altered fibrin clot properties after adjustment for total and or γ' fibrinogen concentration. Aging was associated with thicker fibres ($p = 0.004$) while both metabolic syndrome and low HDL-cholesterol levels were associated with lower rates of lateral aggregation (slope), ($p = 0.0004$ and $p = 0.0009$), and the formation of thinner fibres ($p = 0.007$ and $p = 0.0004$). Elevated CRP was associated with increased rates of lateral aggregation ($p = 0.002$) and consequently thicker fibres ($p < 0.0001$). Hyperhomocysteinemia was associated with increased rates of lateral aggregation ($p = 0.0007$) without affecting fibre thickness.

Conclusion: Final clot structure may contribute to increased CVD risk *in vivo* through associations with other CVD risk factors independent from total or γ' fibrinogen concentration.

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Introduction

Thrombosis, or fibrin clot formation obstructing critically situated blood vessels that cause loss of blood flow to vital organs, is considered the immediate underlying cause of CVD events [1]. Both arterial and venous CVD, have been shown to be accompanied by altered clot structure [2–5]. Furthermore, the architecture of such a fibrin fibre network is important as it affects clot stability, *e.g.* viscoelastic or mechanical properties and fibrinolytic characteristics [6,7]. This eventually determines whether a clot will be lysed successfully or become occlusive or embolise [6,8]. Particularly a clot structure consisting of tightly packed, thin fibres, reduced permeability and characterised by prolonged clot lysis time (CLT), have been associated with CVD events [9] such as stroke [3], myocardial infarction [2], acute coronary syndrome [10] and venous thromboembolism [4].

It is further known that clot structure can be influenced by several factors such as plasma concentration of albumin [11], prothrombin [12], polyphosphates [13] and Factor XIIa [14]; alterations of pH and ionic strength [15]; cross-linking by Factor XIIIa [16,17] as well as plasma total fibrinogen and fibrinogen gamma prime (γ') levels [18]. These factors do not only influence final clot structure, but also the kinetics of clot formation [19,20]. Clot structure is to a large extent kinetically controlled and therefore information on clot formation will aid in the understanding of the final clot structure [21–23]. Increased fibrinogen levels have previously been associated with a shorter lag phase (associated with a tighter fibrin network) [19,21], an increased rate of lateral aggregation (slope) and increased maximum absorbance (indicative of

Abbreviations: CVD, cardiovascular disease; CRP, C-reactive protein; HDL, high density lipoprotein-cholesterol; CLT, clot lysis time; γ', gamma prime; BMI, body mass index; MetS, metabolic syndrome; HbA1c, glycosylated haemoglobin; PURE, Prospective Urban and Rural Epidemiological; Hcy, homocysteine; $PAI\text{-}1_{act}$, plasminogen activator inhibitor-1 activity; hs-CRP, high sensitivity CRP; tPA, tissue plasminogen activator; TF, tissue factor; CI, confidence interval; ANOVA, analysis of variance; ANCOVA, analysis of co variance; IL-6, interleukin-6; WC, waist circumference; EC, endothelial cell; HTL, homocysteine thiolactone; Fbg, fibrinogen.

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thicker fibres) [19–21]. Fibrinogen γ' is associated with a heterogeneous, non-uniform clot structure with prolonged CLT [24,25]. Furthermore, total and γ' fibrinogen have been found to be associated with several known CVD risk factors in this African population [20,26]. Both total and γ' fibrinogen were associated with gender (increased levels in women), abdominal obesity, body mass index (BMI), metabolic syndrome (MetS), glycosylated haemoglobin (HbA1c), CRP and HDL-cholesterol (negatively) [20].

Fibrinogen is thought to contribute to CVD not only through altering the fibrin network structure, but also through several other mechanisms such as increasing platelet aggregation, plasma viscosity and red blood cell aggregation; compromising vascular or endothelial layer function and integrity and participating in the inflammatory process, [27–32].

The purpose of this article was to determine whether the CVD risk factors associated with increased total and/or γ' fibrinogen concentration were also associated with altered fibrin clot properties. The second aim was to investigate whether such associations were due to the fibrinogen concentration itself or whether the CVD risk factors influenced clot properties independently.

Materials and Methods

Study Population

Participants in the study were from the South African arm of the international Prospective Urban and Rural Epidemiological (PURE) study, which is a large cohort study tracking changing lifestyles, risk factors and chronic diseases by using periodic standardised data collection in rural and urban areas of 21 countries in transition over 10 years. Data reported in this study were from the baseline data of 2010 apparently healthy participants that were randomly selected from well-established rural (n = 1006, living under tribal law) and urban (n = 1004, living in informal and formal settlements surrounding cities) study sites in the North West Province of South Africa, collected over a twelve-week period in 2005. Details regarding the selection process and randomisation have been reported elsewhere [1,26]. Inclusion criteria were apparently healthy black South African men and women between the ages of 35 and 65 years. Exclusion criteria were the use of chronic medication for non-communicable diseases and/or any self-reported acute illness. Written informed consent was obtained from participants prior to commencement of the study. The study was approved by the Ethics committee of the North-West University, South Africa.

Blood Collection

Fasting blood samples were collected between 07:00 and 11:00 by qualified nurses with the use of sterile winged infusion sets and syringes with minimum stasis from the antecubital veins of participants. Tubes without anticoagulant were used to collect serum for the analysis of lipids and CRP. To analyse homocysteine (Hcy) and HbA1c, blood samples were collected in EDTA tubes and for glucose measurements in fluoride tubes. Citrated tubes were used for determination of plasminogen activator inhibitor-1 activity ($PAI\text{-}1_{act}$), CLT, total and γ' fibrinogen as well as the turbidimetric measurements of clot formation. Samples were centrifuged at 2000 x g for 15 minutes at 10 °C within 30 minutes of collection. Aliquots were frozen on dry ice, stored in the field at −18 °C and then, after 2–4 days, at −82 °C until analysis.

Laboratory Analysis

The methods for lipids, high sensitivity CRP (hs-CRP), Hcy, $PAI\text{-}1_{act}$, HbA1c and plasma glucose have been described previously [1,26]. Fibrinogen was measured by a modified Clauss method (Multifibrin *U*-test, BCS coagulation analyser, Dade Behring, Deerfield, IL, USA). The analysis of fibrinogen γ' was performed with an ELISA using a 2.G2.H9 mouse monoclonal coating antibody against the human γ' sequence from Santa Cruz Biotechnology (Santa Cruz, USA) for antigen capture and a goat polyclonal horseradish peroxidase–conjugated antibody against human fibrinogen from Abcam for development (Cambridge, USA) [20,33]. Plasma fibrinolytic potential was determined by turbidimetric analysis (A405 nm) according to the method of Lisman et al. [34] and validated by Talens et al. [35]. Tissue-factor-induced plasma clots, lysed by exogenous tissue plasminogen activator (tPA) was used, with slightly modified tissue factor (TF) and tPA concentrations in order to obtain comparable CLTs of about 60 min (intra-assay CV = 3.6%, between plate CV = 4.5%). The final concentrations used were as follows, TF (125x diluted – an estimated final concentration of 59 pM [36]; Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany), $CaCl_2$ (17 mmol/l), tPA (100 ng/ml; Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and phospholipid vesicles (10 μmol/l; Rossix, Mölndal, Sweden). Clot lysis time was defined as the time from the midpoint of the transition from baseline to maximum turbidity, which is representative of clot formation, to the midpoint in transition from maximum turbidity back to baseline, which represents the lysis of the clot [34]. Kinetics of clot formation, *i.e.* lag time, slope, and maximum absorbance were additionally calculated from these curves. Since plasma was clotted with TF, the lag time included the time required for activation of the coagulation cascade as well as the time required for fibrin fibres to grow sufficiently to allow lateral aggregation. The rate of lateral aggregation (slope) was calculated at half maximum absorbance, and the maximum absorbance (indicator of fibre cross-sectional area) was calculated as the maximum absorbance at plateau minus the baseline. The CV for all assays was <10%.

Statistical Analysis

Statistical analyses were performed using the computer software package Statistica (Statsoft, Tulsa, OK, USA). A p-value <0.05 was regarded as statistically significant. Normally distributed data was reported as means [95% confidence interval (CI)] and nonparametric data as geometric mean (95% CI). Nonparametric data was log transformed to improve normality. Independent t-tests and analysis of variance (ANOVA) with Tukey's Honest Significant Difference *post-hoc* tests, were used to compare total and γ' fibrinogen and clot properties across CVD risk factor categories. In order to determine whether the association between clot properties and CVD risk factors were due to differences in total and or γ' fibrinogen, analysis of covariance (ANCOVA) was performed by adjusting for total and or γ' fibrinogen, when they differed significantly across the CVD risk factor categories. These results are reported as least square means (95%CI). Additionally regression models were constructed, using continuous variables, to serve as trend tests to determine the existence of a significant linear trend in the association between the known CVD risk factors and total and γ' fibrinogen and clot properties. The method of Benjamini and Hochberg was used to account for multiple testing [37]. For all significant interactions, significance remained after accounting for multiple testing.

Results

Characteristics of the Study Population

Table 1 provides the descriptive characteristics of the study population. The geometric means for total and γ' fibrinogen concentrations were 3.16 (3.08-3.25) g/l and 0.33 (0.32-0.33) g/l, respectively. Means for measurements of clot formation were as follows, lag time 6.46 (6.37-6.55) minutes, maximum absorbance 0.43 (0.42-0.44), and CLT 57.3 (56.8-57.8) minutes. The geometric mean for slope was 2.44 (2.38-2.49) x 10^{-3} absorbance units/s.

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