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Leukocyte, Haemostatic and Inflammatory Biomarkers Are Activated in Patients with Cardiovascular Disease (CVD): A Pilot Study

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Authors' contributions

This work was carried out in collaboration between all authors. The design of the study, subject recruitment and blood sampling procedures were carried out by authors KLD and SFH. Author KLD performed all of the analytical procedures with help from author JPS during ELISA and flow cytometry testing. Authors SJR, AKB and DTV provided assistance with clinical interpretation. Author SFH provided supervisory support during the study, with authors KLD and SFH drafting the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: Cardiovascular disease (CVD) provides a huge economic strain worldwide and is responsible for over 4 million deaths in Europe annually. Atherosclerosis, a key component of CVD, is recognised as an inflammatory process. This clinical pilot-study aimed to compare a range of selective leukocyte, haemostatic and inflammatory biomarkers in patients with CVD to healthy volunteers.

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Patients and Methods: Fifty participants were recruited, 21 patients with CVD (due to atherosclerosis) and 29 healthy volunteers (with no history of CVD or diabetes). All participants for the study provided a non-fasting venous blood sample prior to analysis (n=50). The biomarkers measured included the Cluster of Differentiation 11b (CD11b) cell surface expression in monocytes and neutrophils, neutrophil elastase, fibrinogen, von Willebrand Factor (vWF), protein C and C-Reactive Protein (CRP).
Results: vWF levels were significantly raised in CVD patients (186.8±106.6 %vWF:Ag) compared to healthy volunteers (109.9±85.2 %vWF:Ag), ($p<0.001$). CRP was significantly raised (3mg/dL) in CVD patients compared to healthy volunteers (<3mg/dL), ($p=0.036$), with the CD11b cell surface expression in monocytes being higher (0.64±0.55 MFI) in CVD patients compared to healthy volunteers (0.37±0.44 MFI), ($p<0.005$). No differences were observed for protein C, fibrinogen, neutrophil elastase or neutrophil CD11b in CVD patients compared to healthy volunteers ($p>0.05$).
Discussion and Conclusion: Patients with CVD have elevated levels of vWF, CRP and CD11b cell surface expression (monocytes) compared to healthy volunteers. The results of this study support the premise of leukocyte, haemostatic and inflammatory involvement during CVD, and that measuring biomarkers such as vWF and leukocyte CD11b cell surface expression, may aid in the diagnosis and monitoring of patients with CVD. However, further large-scale prospective studies are required to fully understand the relationship between these markers and CVD.

Keywords: Leukocytes; haemostasis; inflammation; cardiovascular disease.

ABBREVIATIONS

CVD- Cardiovascular Disease; CRP- C-Reactive Protein; vWF- von Willebrand Factor; EDTA- di-potassium ethylene diamine tetra-aceticacid; ELISA- Enzyme Linked Immunosorbent Assay; MFI- Mean Fluorescence Intensity; IL- Interleukin; CD11b - Cluster of Differentiation 11b; PBS- Phosphate Buffered Saline; MI- Myocardial Infarction; AF- Atrial Fibrillation; CIMT- Carotid Artery Intima-Media Thickness; CHD- Coronary Heart Disease.

1. BACKGROUND

Cardiovascular disease (CVD) provides a huge economic strain worldwide and is responsible for over 4 million deaths in Europe annually [1]. Atherosclerosis, a recognised risk factor for CVD, has been extensively described as an inflammatory process [2,3,4]. Atherosclerotic lesions occur primarily in elastic and muscular arteries, and can lead to ischaemia of the brain, heart or extremities, resulting in infarction [3]. The inflammatory nature of atherosclerosis in CVD has led to interest in assessing various leukocyte, haemostatic and inflammatory markers for monitoring and diagnosing CVD [4].

One of the most studied biomarkers in CVD is C-Reactive Protein (CRP), an acute phase protein elevated in infection and inflammation. CRP is a strong, independent risk factor for predicting future cardiovascular events [5]. CRP is present within atherosclerotic plaques, and can be produced by smooth muscle cells, leading to elevated CRP levels in patients with CVD [6]. Casula et al. [7] reported that minor increases in CRP were strong, independent predictors of cardiovascular events. Urbonaviciene et al. [8] reported that high-sensitivity-CRP (hs-CRP) was associated with cardiovascular death in patients with

peripheral arterial disease (PAD), but that other inflammatory markers should be used alongside hs-CRP to reliably determine prognosis.

Fibrinogen is another marker which has demonstrated significance in patients with CVD. Atherosclerotic plaques contain large quantities of fibrin, fibrinogen and fibrinogen degradation products, and fibrinogen elevation is associated with increased CVD risk [9]. Green et al. [10] reported a significant positive association between high fibrinogen and carotid artery intima-media thickness (CIMT), which is a determinant of asymptomatic atherosclerosis. Espinola-Klein et al. [11] reported that fibrinogen was an independent predictor of cardiovascular mortality, atherosclerotic burden and prognosis.

von Willebrand factor (vWF) is a large glycoprotein which mediates platelet adhesion and is produced in vascular endothelial cells in response to vascular damage. This has led to interest in vWF levels in patients with CVD. Freynhofer et al. [12] reported that patients with atrial fibrillation (AF) had higher levels of vWF than normal controls, suggesting vWF had a causal role in CVD and in atherosclerosis. Elevated vWF levels indicate on-going inflammatory, endothelial and haemostatic activation in patients with CVD [13].

There are a range of other biomarkers which have not been extensively studied in CVD patients including protein C, cluster of differentiation 11b (CD11b) and neutrophil elastase. As these biomarkers have recognised functions in leukocyte and haemostatic activation they could be considered for the diagnosis and monitoring of CVD. Protein C is a plasma glycoprotein involved in the coagulation cascade during haemostasis, which also has an anti-inflammatory role [14]. Lay et al. [15] used murine models of protein C deficiency, and demonstrated a hypercoagulable and hyper-inflammatory state, supporting an anti-inflammatory role of protein C by inhibiting thrombin generation and down regulating inflammatory mediators such as interleukin-6 (IL-6).

CD11b is a member of the α-chain integrin family, expressed predominantly on neutrophils, monocytes and natural killer cells, and facilitates leukocyte rolling along the endothelium in the leukocyte adhesion cascade. Liu et al. [16] reported significantly increased CD11b levels in patients with acute myocardial infarction (MI), and suggested that increased neutrophil CD11b expression is intrinsically associated with atherosclerosis. Liu et al. [17] demonstrated a significant increase in both neutrophil and monocyte CD11b in MI patients with a coronary stent, and reported that CD11b expression was related to the severity of the MI.

Neutrophil elastase is an effector molecule of the innate immune system contained within azurophilic granules in neutrophils, which degrades products of the extra cellular matrix. Neutrophil elastase has been reported as a biological marker of leukocyte activation [18]. Atherosclerotic plaques which are vulnerable to rupture contain high levels of neutrophil elastase [19]. El-Eshmawy et al. [20] determined neutrophil elastase levels in a group of obese pre-hypertensive women, a known CVD risk factor, and concluded that elastase levels were significantly higher than in healthy volunteers.

This clinical pilot-study aimed to test the hypothesis that patients with CVD due to atherosclerosis express increased leukocyte, haemostatic and inflammatory activation compared to healthy volunteers.

2. METHODS

2.1 Subject Volunteers

Ethical approval was obtained from the local research ethics committee (West Midlands-Solihull Reference Number: 13/WM/0170).

21 patients were recruited at random from the CVD clinic at Russells Hall Hospital, and all had CVD due to atherosclerosis (n=21). Specifically, the CVD group consisted of seven females and 14 males, aged 36-79 years (mean age 64.55 years). 15 out of the 21 participants declared that they were taking statins to treat hypercholesterolemia. In addition, 6 participants in the CVD group were taking aspirin. In this group, 16 participants were current or former smokers, and 14 participants were aware that they had a history of high cholesterol. The majority of participants (18) had suffered a cerebral event (a stroke or transient ischaemic attack (TIA)).

The healthy control group consisted of 23 females and 6 males, aged 21-61 years (mean age 34.85 years), with none of the participants having any history of CVD or diabetes, and none were taking statins or aspirin (n=29).

2.2 Blood Samples

All participants provided a non-fasting venous blood sample and completed a standard questionnaire relating to lifestyle and medical history. Venous blood samples were collected into various Greiner Vacuette® tubes (i.e. tri-sodium citrate, serum clot activator and di-potassium ethylenediaminetetra-acetic acid (EDTA)).

2.3 Measurement of Leukocyte Activation (CD11b and Neutrophil elastase) CD11b

CD11b was determined using the Beckman Coulter FC500 flow cytometer, using a di-potassium EDTA sample. The monoclonal antibody used was Coulter Clone® Mo1-FITC, mouse IgM antibody, with IOTest mouse IgM-FITC isotype control clone GC323, supplied by Beckman Coulter. This method was previously described by Li et al. [21]. 100µl of whole blood was incubated for 10 minutes at room temperature with 5µl of working antibody solution (5µl stock antibody and 195µl Phosphate Buffered Saline (PBS)). 100µl of whole blood was also incubated with 20µl of isotype control under the same conditions. 500µl of Optilyse® C was added to each tube, vortexed and then incubated for 10 minutes. 500µl of PBS was added to each tube and incubated for 5 minutes, then centrifuged at 2500 rpm for 5 minutes. The supernatant was removed and the pellet re-suspended in 1mL of PBS. The solution was vortexed before analysis with pre-determined gating for monocytes and neutrophils.

2.4 Neutrophil Elastase

Neutrophil elastase was measured using an enzyme-linked immunosorbent assay (ELISA) kit supplied by Hycult Biotech (Netherlands). Blood collected into a di-potassium EDTA tube was placed on ice and centrifuged at 3000 r.p.m. for 10 minutes. Plasma was removed and stored at -70ºC until required for analysis. ELISA plates were incubated with 100µl of

standard and samples for 1 hour at room temperature, and then washed 4 times. 100µl of diluted tracer was added to each well and the plate was incubated for one hour at room temperature, and then washed 4 times. 100µl of diluted streptavidin-peroxidase was added to each well, and incubated for 1 hour at room temperature and subsequently washed 4 times. 100µl of tetramethylbenzidine (TMB) substrate was then added to each well and incubated for 30 minutes at room temperature (protected from sunlight). 100µl of stop solution was added and the plate was read at 450nm.

2.5 Measurement of Haemostatic Activation (Fibrinogen, vWF and Protein C) Fibrinogen

Fibrinogen was measured using the Instrumentation Laboratories ACL-TOP® automated haemostasis analyser, using a tri-sodium citrate sample centrifuged for 10 minutes at 3000 r.p.m. The assay was performed using the RecombiPlasTin 2G reagent kit, as previously described by Mackie et al. [22]. Normal and abnormal quality control samples were performed prior to sample analysis.

2.6 vWF and Protein C

Protein C and vWF were measured using blood collected in tri-sodium citrate, employing the Instrumentation Laboratories ACL-TOP® analyser. vWF antigen was determined using an immuno-turbidimetric assay. Protein C was quantified following incubation with a protein C activator, and determined by measuring the quantity of chromogenic substrate produced at 450nm. Both methods were previously described by Appert-Flory et al. [23]. Normal and abnormal commercial controls were performed prior to processing patient samples.

2.7 Measurement of Inflammatory Activation (CRP) CRP

CRP was determined using the VITROS® CRP slide method on the VITROS®5,1 FS analyser. Blood collected into serum clot activator tubes was centrifuged at 4500 r.p.m. for 5 minutes. The assay is based on an enzymatic heterogeneous sandwich immunoassay, where a phosphorylcholine (PC) derivative is covalently bound to polystyrene polymer beads. This method has been previously described by Lelong et al. [24]. Quality control specimens were processed prior to patient samples.

2.8 Statistical Analysis

Data analysis was performed using SPSS software, version 22.0. Where data were normally distributed, a student t-test was employed adopting a 5% level of significance. Data that did not comply with normality was analysed using the Mann-Whitney test, with statistical significance accepted when $p \leq 0.05$.During this study, all results were presented as mean ± standard deviation (SD) or median ± Iqr, where n indicates the number of participants.

3. RESULTS

3.1 Measurement of Leukocyte Activation (CD11b and Neutrophil Elastase) CD11b

Patients with CVD expressed higher neutrophil CD11b cell surface expression (1.20±0.59 mean fluorescence intensity (MFI)) in comparison to that of healthy volunteers (0.90±0.70 MFI), although these were not significantly different as determined by the Mann-Whitney test ($p>0.05$).With regards to monocyte CD11b cell surface expression, patients with CVD expressed significantly higher CD11b (0.64±0.55 MFI) in comparison to healthy volunteers (0.37±0.44 MFI), as determined by the Mann-Whitney test ($p=0.005$), (Figs. 1A+B).

3.2 Neutrophil Elastase

There were reduced levels of neutrophil elastase in the CVD group (80.76±44.84ng/mL) when compared to the healthy volunteers (94.00±47.07ng/mL), although these changes were not significant as determined by the Mann-Whitney test ($p>0.05$), (Fig. 2).

3.3 Measurement of Haemostatic Activation (Fibrinogen, vWF and Protein C) Fibrinogen

Fibrinogen levels in the CVD patient group were higher (3.88±0.36 mg/dL) than in the healthy volunteers group (3.75±1.04 mg/dL). However, these differences were not significant as determined by the Mann-Whitney test ($p>0.05$), (Fig. 3).

3.4 vWF

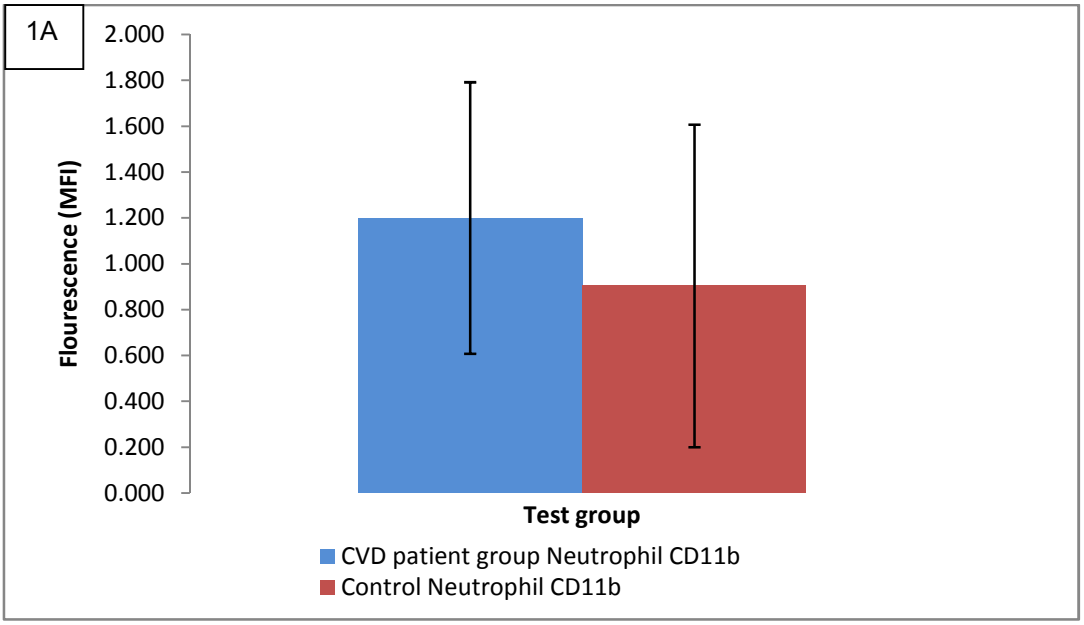
Patients with CVD demonstrated significantly higher levels of vWF (186.8±106.6 %vWF:Ag) in comparison to healthy volunteers (109.9±85.2 %vWF:Ag), as determined by the Mann-Whitney test ($p<0.001$), (Fig. 4).

3.5 Protein C

Patients with CVD demonstrated a lower protein C level (98.86±33.09% activity) in comparison to healthy volunteers (109.62±23.09 % activity), although these changes were not significant as determined by the Student t-test ($p>0.05$), (Fig. 5).

3.6 Measurement of Inflammatory Activation (CRP) CRP

Patients with CVD demonstrated significantly higher CRP levels (3±<3-4.5mg/dL) compared to healthy volunteers <3±<3-<3mg/dL), as determine by the Mann-Whitney test ($p=0.036$), (Fig. 6).



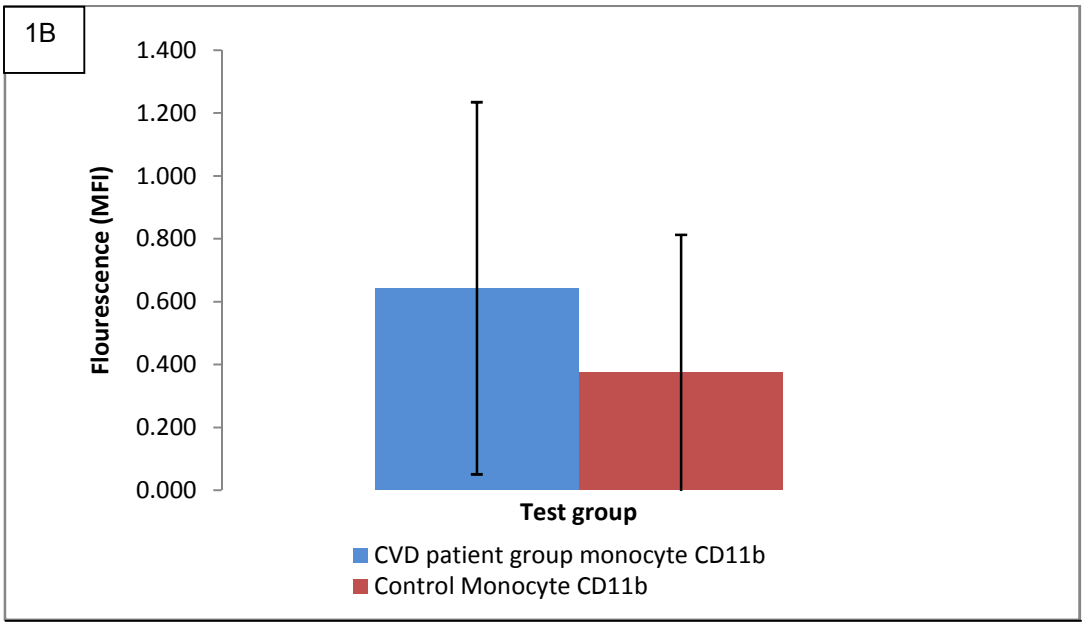


Fig. 1. (A) Neutrophil CD11b (MFI) levels in CVD patients (n=21) compared to normal controls (n=29).The points represent median± Iqr. p>0.05 as determined by the Mann-Whitney test. (B) Monocyte CD11b (MFI) levels CVD patients (n=21) compared to normal controls (n=29). The points represent median ± Iqr. p=0.005 as determined by the Mann-Whitney test

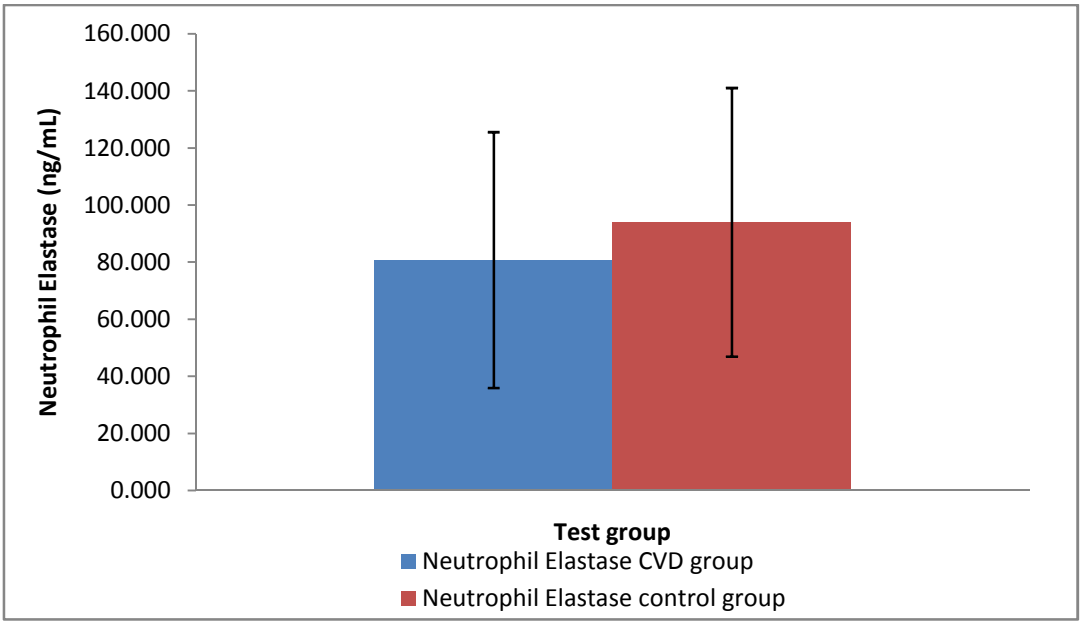


Fig. 2. Neutrophil elastase (ng/mL) levels in CVD patients (n=21) compared to healthy volunteers (n=29). The points represent median± Iqr. p>0.05 as determined by the Mann-Whitney test

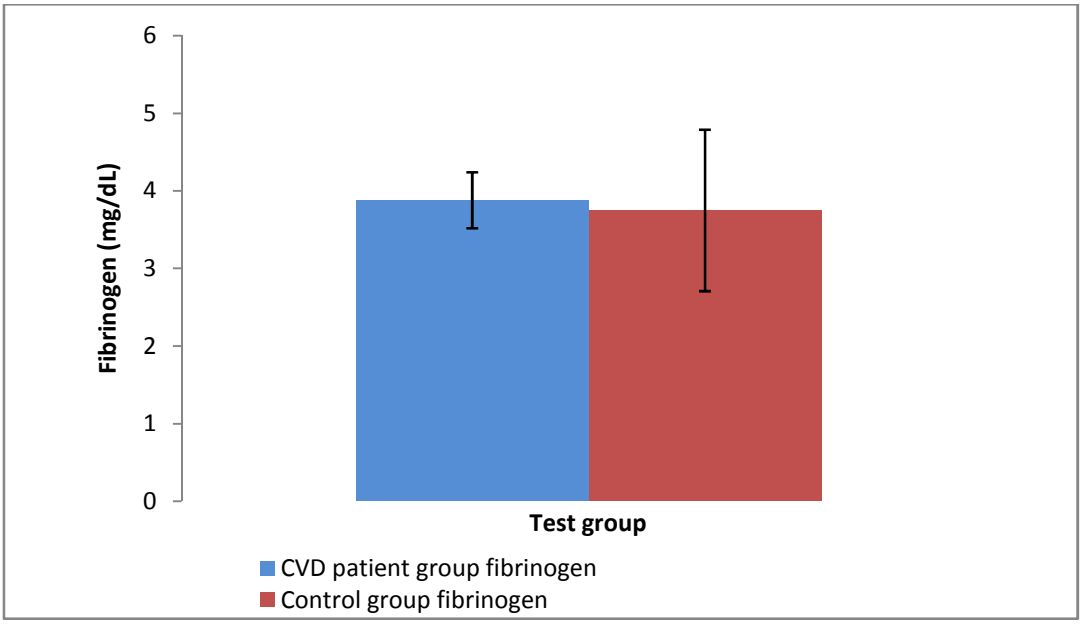


Fig. 3. Fibrinogen (mg/dL) levels in CVD patients (n=21) compared to healthy volunteers (n=29). The points represent median± Iqr. p>0.05 as determined by the Mann-Whitney test

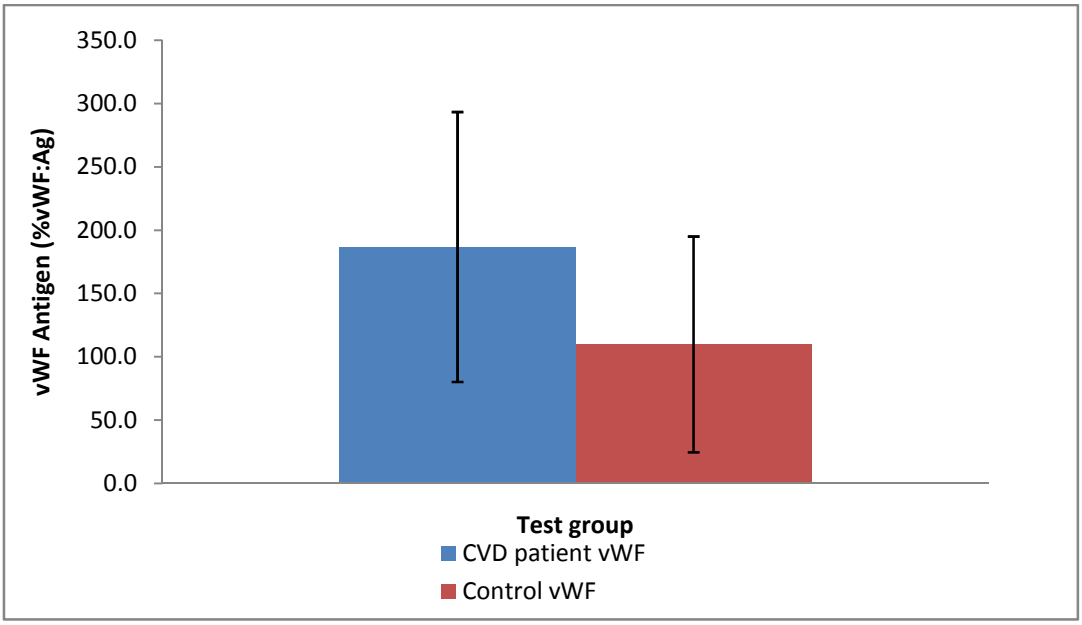


Fig. 4. vWF (%vWF:Ag) levels in CVD patients (n=21) compared to healthy volunteers (n=29). The points represent median± Iqr. p<0.001 as determined by the Mann-Whitney test

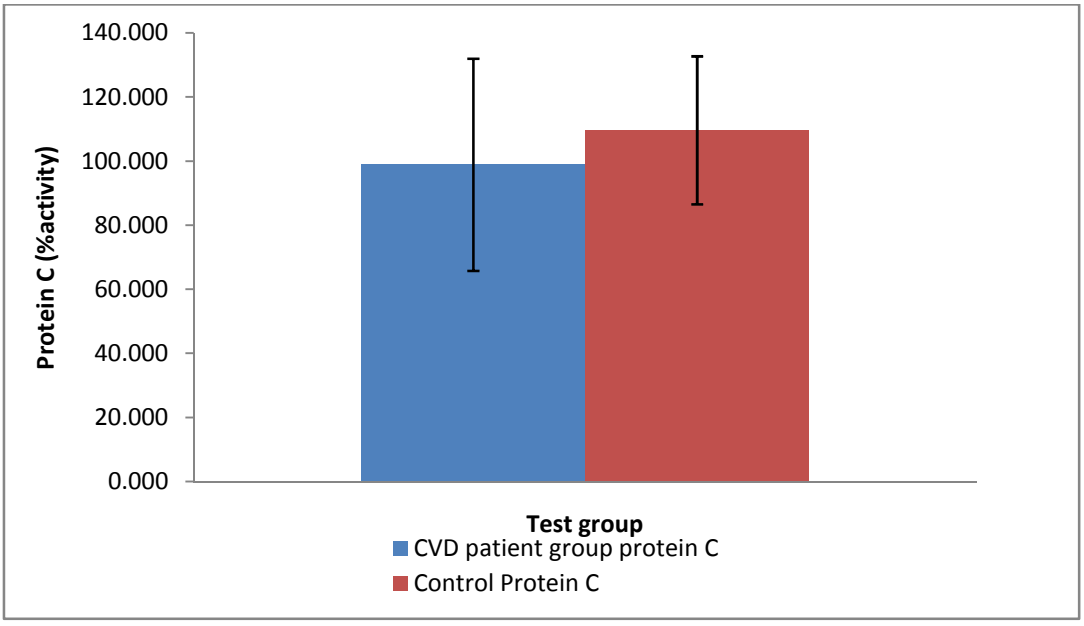


Fig. 5. Protein C (%activity) levels in CVD patients (n=21) compared to healthy volunteers (n=29). The points represent mean ±standard deviation. p>0.05 as determined by a Student t-test

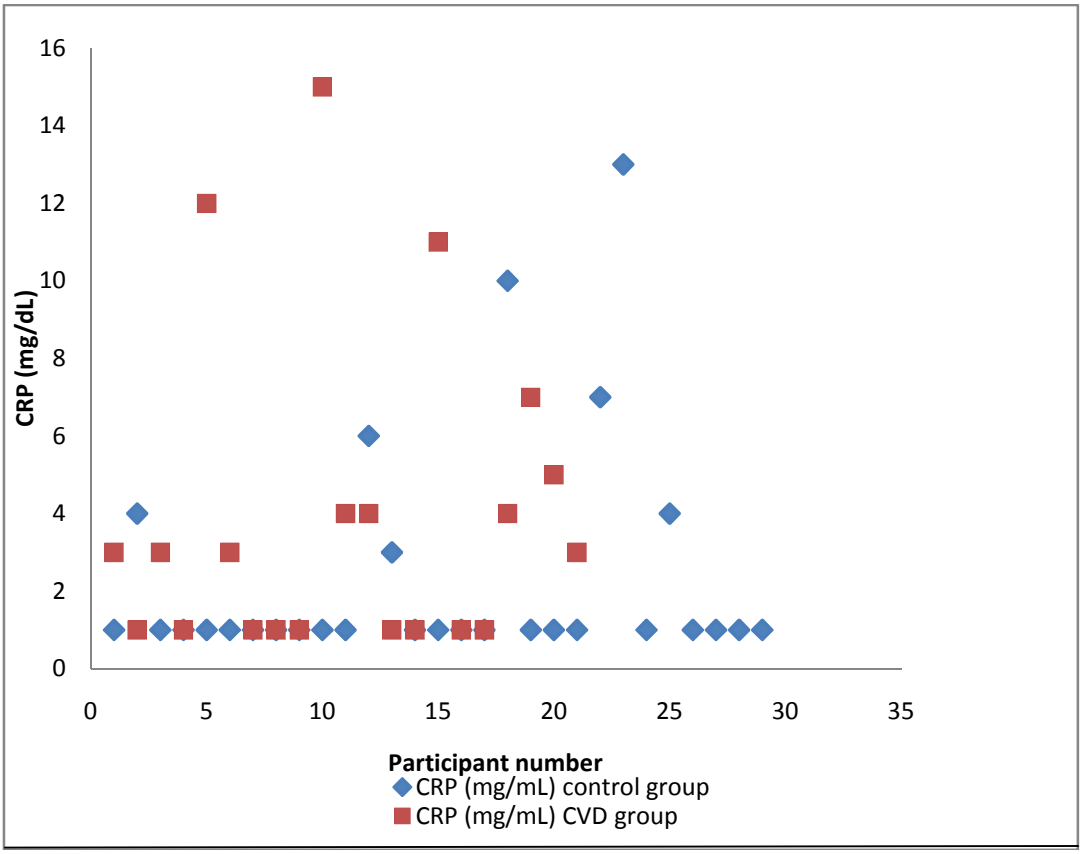


Fig. 6. CRP levels (mg/dL) in CVD patients (n=21) compared to healthy volunteers (n=29). CRP levels in patients with CVD were found to be significantly higher than in controls as determined by a Mann-Whitney test (p=0.036)

4. DISCUSSION

This clinical pilot-study aimed to compare a range of selective leukocyte, haemostatic and inflammatory biomarkers in patients with CVD due to atherosclerosis compared to healthy volunteers with no history of CVD and diabetes. Of the biomarkers investigated, there was a significant difference between the CD11b cell surface expression in monocytes, vWF and CRP in patients with CVD compared to healthy volunteers ($p=<0.05$).

In CVD patients, vWF was significantly elevated compared to healthy volunteers ($p<0.001$). Other studies [10,25] have reported an increase in vWF concentration in patients with CVD, including Freynhofer et al. [12], who reported that vWF levels were significantly higher in patients with atrial fibrillation. The elevated vWF levels in patients with CVD demonstrate an on-going inflammatory, endothelial and haemostatic activation [13]. In agreement with Skeppholm et al. [13] our study provides further evidence of endothelial and haemostatic activation in CVD patients in comparison to healthy volunteers.

Leukocyte CD11b facilitates leukocyte-endothelial cell interactions by promoting leukocyte rolling along the endothelium [26]. Monocyte CD11b cell surface expression was significantly

elevated in the CVD group compared to healthy controls (p=0.005). There was also a trend of increasing neutrophil CD11b cell surface expression in the CVD group compared to healthy volunteers (p>0.05). Liu et al. [16] measured CD11b levels in Coronary Heart Disease (CHD) patients following coronary stent insertion, a procedure associated with vascular damage and potential rupture of atherosclerotic plaques, and reported significantly increased levels of monocyte and neutrophil CD11b. Increased CD11b levels result in greater adhesion to ligands on the vascular endothelium, which can cause micro-vascular damage, suggesting a possible mechanism for CD11b involvement in CVD [17]. Results from the present study support findings from other studies, and provides additional provision to consider routinely measuring this parameter as a potential marker to aid in the diagnosis or monitoring of CVD.

CRP, an acute phase protein which is elevated in response to inflammation or infection, was significantly raised in CVD patients when compared to healthy volunteers (p=0.036). The findings of this study agree with Gupta et al. [5] who reported that measuring baseline CRP was an independent predictor of cardiovascular events. Sharma et al. [27] reported that CRP could predict recurring cardiovascular events in patients who had suffered a previous MI. Our findings support the observations of previous studies, those of significantly raised CRP levels in CVD patients, and clarify the chronic inflammatory state associated with CVD.

Fibrinogen is a circulating coagulation protein, which also acts as a non-specific acute phase reactant in inflammation. In the present study there was a trend of increasing fibrinogen concentration in the CVD group when compared to healthy volunteers, although this did not reach statistical significance (p>0.05). Atherosclerotic plaques contain large quantities of fibrin and fibrinogen, suggesting a direct role of fibrinogen in the initiation of atherogenesis and growth of atherosclerotic lesions [9]. Green et al. [10] reported that fibrinogen may be used as a potential marker of atherosclerosis. Although not significant, the findings of increased fibrinogen levels in CVD patients compared to healthy volunteers in the present study, support the premise that fibrinogen may be used as a marker for atherosclerosis and subsequently CVD. The failure of fibrinogen to reach statistical significance in the present study, is probably due to small sample size. Larger numbers of participants would be required to fully establish the suitability of this parameter as a marker of atherosclerosis and CVD.

Protein C has a recognised function in haemostasis, specifically during the coagulation cascade, but also has anti-inflammatory properties. Esmon [14] reported that protein C levels were significantly lower in patients with inflammatory conditions when compared to healthy volunteers, due to the recognised anti-inflammatory role of protein C. In the present study, a decreasing protein C concentration was observed in CVD patients when compared to healthy volunteers, although these were not significant. Our findings are in agreement with Esmon [14] and Esmon [28], and provides a sound platform to continue work on this parameter as studies involving protein C in patients with CVD are limited.

In this clinical pilot-study, patients with CVD demonstrated reduced neutrophil elastase concentration when compared to healthy volunteers (p>0.05). Neutrophil elastase is a proteolytic enzyme, which contributes to the rupture of vulnerable atherosclerotic plaques as it breaks down the extracellular matrix [29]. Vulnerable atherosclerotic plaques, with thin fibrous caps contain higher levels of neutrophil elastase than fibrous plaques and normal arteries [30]. El-Eshmawy et al. [20] demonstrated significantly increased levels of neutrophil elastase in obese women with pre-hypertension, a known CVD risk factor. The findings from the present study did not provide significant changes in elastase between the two groups

($p>0.05$), and the lower levels of neutrophil elastase in the CVD population was not expected. A possible explanation for this is that the treatment (statins) which the CVD patients were taking, may have had an impact on leukocyte function, thus reducing the levels of neutrophil elastase observed in the present study. However, to fully establish this further studies would need to be undertaken.

The present study did have some limitations, particularly that more participants could have been recruited for the study. Based upon the statistical power calculation, 110 participants were required for each group, but due to time constraints only 21 CVD participants and 29 healthy volunteers were recruited. The participants in the CVD group were relatively stable, and had been recovering from their cardiovascular event for several months, although the precise duration between the events and participation is unknown. If participants were recruited shortly after a cardiovascular event, this may have resulted in more dramatic increases in the markers which were being investigated. There are other conditions, such as hypertension and diabetes which may also effect the biomarkers considered here, and this should be addressed in further large-scale prospective studies. The control group is not matched with the CVD group for age or gender and this should also be addressed in future studies. Despite the limitations associated with this study, our results indicate that specific biomarkers such as vWF, CD11b and CRP have the potential to be incorporated into routine screening methods currently available for the diagnosis of CVD.

5. CONCLUSION

This study aimed to compare a range of leukocyte, haemostatic and inflammatory biomarkers in patients with CVD compared to normal healthy volunteers. Our study demonstrated that patients with CVD showed significantly increased levels of vWF ($p<0.001$), CD11b cell surface expression in monocytes ($p=0.005$) and CRP ($p=0.036$) compared to healthy volunteers. Findings from our study demonstrate observable changes in selective biomarkers such as vWF, between patients with atherosclerosis and healthy volunteers. This provides a sound platform for further studies, although differences due to atherosclerosis would need to be determined with comparison to an age/sex matched control group free of atherosclerosis. This study supports the possible benefits of measuring specific biomarkers such as CD11b, as early diagnostic tools for monitoring or predicting the risk for patients of developing CVD.

CONSENT

All authors declare that written informed consent was obtained from all participants for publication of this data.

ETHICAL APPROVAL

Ethical approval (Re: 13/WM/0170) for this study was permitted by the local research ethics committee, West Midlands (Solihull).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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