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Markers of Coagulation Activation, Endothelial Stimulation and Inflammation in Patients with Peripheral Arterial Disease

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Objectives. *Patients with peripheral arterial disease have a significantly increased risk of cardiovascular and cerebrovascular mortality. Studies have shown that some haemostatic and inflammatory markers are elevated in these patients but the effect of the severity of the disease has not been fully documented. The aim of this study was to assess the level of coagulation activation, endothelial stimulation and inflammation in patients with claudication and critical limb ischaemia (CLI) compared to healthy controls.*

Design and methods. *A prospective observational study was conducted amongst 202 subjects: 132 claudicants, 30 patients with critical ischaemia, and 40 controls. D-dimer (DD) and thrombin–antithrombin III (TAT) levels measured using ELISA as markers of coagulation activation. von Willebrand factor (vWF) and high-sensitivity C-reactive protein (CRP) levels were measured as markers of endothelial and inflammatory stimulation.*

Results. *vWF and CRP levels were significantly higher in patients with intermittent claudication (1.9 U/ml, range 0.78–4.05; $p<0.001$; 3.4 mg/l, range 0.15–24; $p>0.001$, respectively) and critical ischaemia (2.36 U/ml; range 1.03–5.69; $p<0.001$; 7.17 mg/ml, range 0.15–174; $p<0.001$, respectively) compared to controls (1.28 U/ml, range 0.62–3.13; 1.04, range 0.15–7.59 mg/l). DD was also significantly higher in claudicants (48.6 μg/ml; range 2–1741; $p<0.001$) and in patients with CLI (61.1 μg/ml, range 3.65–1963; $p<0.001$) compared to controls (26.1 μg/ml, range 9.65–203.1). TAT levels were significantly higher in CLI (3.14 mg/l, range 2.09–58.11), compared to controls (2.36 mg/l, range 1.49–7.38; $p=0.004$). Patients with CLI had significantly higher levels of CRP, vWF, and TAT than claudicants.*

Conclusions. *Coagulation activation and endothelial stimulation are significantly increased in patients with peripheral arterial disease compared to healthy controls. Coagulation and endothelial activation increases with the severity of the arterial disease.*

Keywords: Haemostasis; Thrombosis and vascular biology; Peripheral arterial disease.

Introduction

Patients with peripheral arterial disease have a significantly increased mortality due to cardiovascular and cerebrovascular events.[1–3] The underlying pathophysiological process leading to these events is atherothrombosis and thromboembolism.[4,5] This involves endothelial damage and changes at the site of atherosclerosis leading to platelet adhesion, aggregation and activation together with activation of coagulation.[6] The end product of this process is occlusive thrombus which leads to ischaemia of the organ supplied.[7]

We have recently shown platelet activation to be increased in patients with intermittent claudication and critical limb ischaemia compared to healthy controls.[8]

Whether, this increased level of activation is primary or secondary to the atherosclerotic changes in these patients is uncertain. Patients with peripheral arterial disease are known to have widespread atherosclerotic changes in the coronary and cerebral vessels which mirror the changes observed in the peripheral vasculature.[3]

In addition to platelet activation, endothelial and coagulation activation have been implicated in the increased incidence of ischaemic events in patients with peripheral arterial disease.[8–15] However, whereas

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anti-platelet therapy is now routine for patients for peripheral arterial disease the use of anti-coagulants is less extensive.[16] Many large trials have shown that in patients with peripheral arterial disease the use of the anti-platelet agent aspirin is associated with a 25% risk reduction of further serious adverse events.[16] Few studies have examined the role of anticoagulants in patients with peripheral arterial disease.

Several studies have shown that haemostatic markers are elevated in patients with peripheral arterial disease.[10–15] However, these studies are mainly in claudicants and the effect of the severity of the disease has not been fully documented and further research is required in this area. Furthermore, little is known about the status and role of inflammatory markers such as CRP in patients with peripheral arterial disease. The aim of this study was to assess the level of coagulation activation and endothelial stimulation in patients with intermittent claudication and critical limb ischaemia compared to healthy controls.

Methods

Participants to the study were recruited from patients referred to the Vascular Unit, Aberdeen Royal Infirmary. Patients with a history and clinical evidence of intermittent claudication presenting at the Vascular clinic were recruited to the study. The diagnosis of intermittent claudication was confirmed by ankle–brachial pressure index (<0.9) and duplex evidence of peripheral vascular disease. Patients with tissue loss (gangrene or ulceration) of the lower limbs secondary to ischaemia admitted to the vascular unit were recruited. Confirmation of severe limb ischaemia as the cause of tissue loss was made by measurement of ankle–brachial pressure index (<0.5) and duplex evidence of peripheral arterial disease. All patients with claudication or critical limb ischaemia were receiving 75 mg aspirin at recruitment.

Healthy controls were recruited from patients referred to the Vascular clinic with varicose veins and who had no history of ischaemic heart disease, cerebrovascular disease or peripheral arterial disease. All controls had normal ankle–brachial pressure indices. None of the controls was receiving aspirin.

Informed consent was obtained from each subject and full ethical approval for the study was sought and granted by the Grampian Research Ethics Committee. Exclusion criteria: patients were excluded if their haemoglobin was less than 100 g/l, platelet count less than 150×10^9/l, creatinine more than twice the upper limit of normal, aspartate aminotransferase, alkaline phosphatase or gamma glutamyl transferase more than three times the upper limit of normal and if the body mass index exceeded 33. Patients with a history of haematological malignancy, acute illness unrelated to peripheral arterial disease within 14 days, transfusion of whole blood within 14 days, known or suspected alcohol or drug abuse or on warfarin, steroids or clopidogrel were also excluded.

Blood investigations

Blood samples were withdrawn from the antecubital vein of rested subjects by clean venpuncture and plasma was stored at $-80\,^{\circ}C$ until assayed. All samples were done in duplicate, with double standards and quality control. Von Willebrand factor (vWF) and high sensitivity C-reactive protein (CRP) were measured as markers of endothelial and inflammatory stimulation. D-dimer and thrombin–antithrombin III (TAT) levels were measured as markers of coagulation activation. vWF was measured by an in-house ELISA using polyclonal rabbit-antihuman vWF antibody and horse radish peroxidase-conjugated antibody from Dako Ltd, Denmark.[17] The inter-assay variation varied between 1.3 and 8.6%, whereas the inter-assay coefficient of variation ranged between 2.9 and 10.1%.

High sensitivity CRP was measured using an immunoturbidimetric assay (Dade Behring, France) with high sensitivity at the Biochemistry laboratory, Aberdeen Royal Infirmary. The coefficient of variation ranged between 1.2 and 2.68%.

TAT complex levels were measured using an ELISA kit (Enzygnost TAT micro, Dade Behring Marburg GmbH, Marburg, Germany). This covered a concentration range of 2–60 μg/l. The intra-assay coefficient of variation fell between 4 and 6% whereas the inter-assay coefficient of variation was between 6 and 9%.

D-dimer was measured using an ELISA kit (Dimertest Gold EIA kit, Agen) with an intra-assay coefficient of variation ranging from 3.7 to 6.8%. The inter-assay coefficient of variation ranged from 6.6 to 10.1%.

Statistical analysis

Results are presented as median values with range. Calculations were performed using SPSS for Windows version 10.0 statistical software. Differences between controls, claudicants and patients with critical limb ischaemia were analysed using the Mann–Whitney *U* test. A *p*-value of less than 0.05 was considered significant.

Results

Subjects

Two hundred and two subjects were recruited to the study, 132 patients with intermittent claudication, 30 patients with critical limb ischaemia and 40 controls. The mean age was 52.8 years for controls, 65.8 years for claudicants and 72.1 years for patients with critical ischaemia. Table 1 shows the sex distribution, smoking habits and other demographic details of subjects recruited.

Coagulation activation and endothelial stimulation in peripheral arterial disease compared to controls

Figs. 1–4 show the results for D-dimer, TAT, CRP and vWF in controls, claudicants and criticals. Results are shown as median values with 25th and 75th percentiles. CRP and vWF were significantly elevated in patients with intermittent claudication (3.4 mg/l, range 0.15–24, $p<0.001$; 1.9 U/ml, range 0.78–4.05, $p<0.001$, respectively) compared to controls (1.04 mg/l, range 015–7.59 and 1.28 U/ml, range 0.62–3.13, respectively). Similarly, both CRP and vWF were significantly higher in patients with critical ischaemia (7.17 mg/l, range 0.15–174, $p<0.001$; 2.36 U/ml, range 1.03–5.69, $p<0.001$, respectively) compared to controls. Furthermore both CRP ($p=0.001$) and vWF ($p=0.005$) were significantly higher in patients with critical ischaemia compared to patients with intermittent claudication.

D-dimer was found to be significantly higher in both patients with intermittent claudication (48.6 μg/ml, range 2–1741, $p<0.001$) and those with critical ischaemia (61.1 μg/ml, range 3.65–1963, $p<0.001$) compared to controls (26.1 μg/ml, range 9.65–203.1). The difference in D-dimer between claudicants and criticals, however, failed to reach statistical significance ($p=0.08$). Similarly, TAT levels were found to be significantly elevated in patients with critical ischaemia (3.14 μg/l, range 2.09–58.11, $p=0.004$) compared to controls (2.36 μg/l, range 1.49–7.38). There was no significant difference, however, between claudicants (2.41 μg/l, range 1.24–63.22, $p=0.48$) and controls in TAT levels.

Table 1. Basic demographics of patients in the three groups

	Controls ($n=40$)	Claudicants ($n=132$)	Criticals ($n=30$)
Sex ratio (M:F)	2:3	3.4:1	1.3:1
Smoking *n* (%)			
Current	10 (25)	45 (34.1)	13 (40.6)
Stopped <1 year	0 (0)	24 (18.2)	3 (9.4)
Stopped >1 year	15 (37.5)	55 (41.7)	13 (40.6)
Never smoked	15 (37.5)	8 (6.1)	3 (9.4)
Diabetics *n* (%)	0 (0)	23 (17.4)	14 (43.8)
History of CHD	0	20 (15.2%)	7 (23%)
Treated hypertension	0	51 (38.6%)	12 (40%)
Age/years (STD)	52.7 (13.9)	65.7 (8.4)	73.2 (8.7)

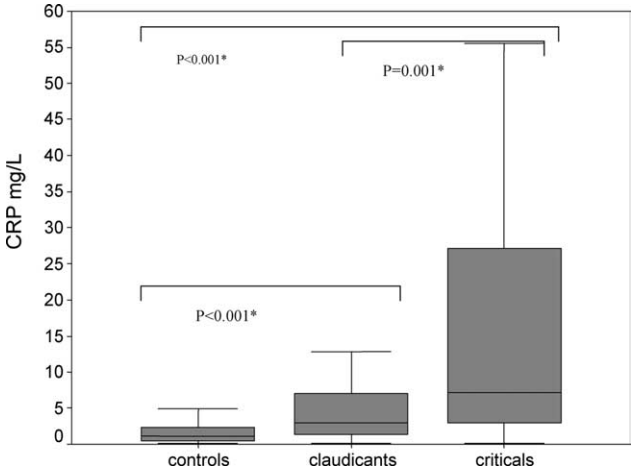

Fig. 1. Boxplots of C-reactive protein levels in controls, claudicants and criticals. (Bars = median values; Whiskers = smallest and largest values excluding extremes; top of box + 75th percentile; bottom of box = 25th percentile). *Statistically significant difference.

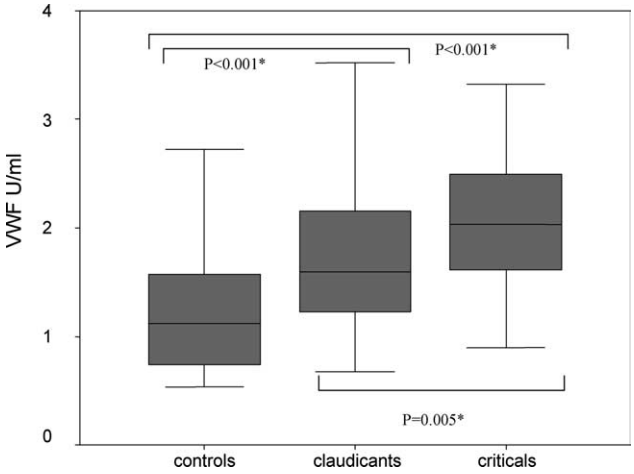

Fig. 2. Boxplots of von Willebrand factor levels in controls, claudicants and criticals. (Bars = median values; Whiskers = smallest and largest values excluding extremes; top of box + 75th percentile; bottom of box = 25th percentile). *Statistically significant difference.

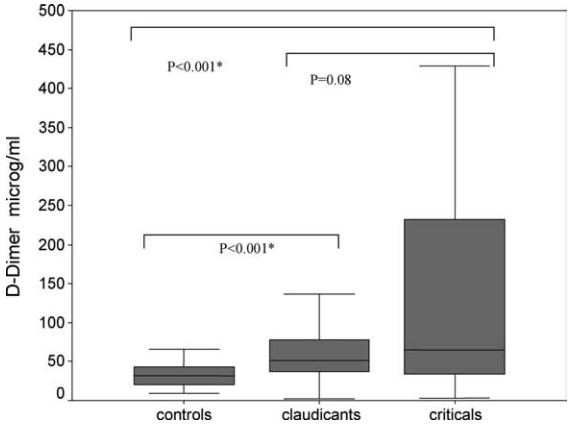

Fig. 3. Boxplots of D-dimer levels in controls, claudicants and criticals. (Bars = median values; Whiskers = smallest and largest values excluding extremes; top of box + 75th percentile; bottom of box = 25th percentile). *Statistically significant difference.

Discussion

This study demonstrates that patients with peripheral arterial disease have enhanced coagulation activation as well as evidence of on going endothelial activation compared to controls. This may be associated with the increased risk of cardiovascular and cerebrovascular events observed in this group of patients. The results also show that the more severe the extent of peripheral arterial disease, the more coagulation activation and endothelial activation is observed. Hence, patients with critical ischaemia had significantly higher levels of CRP, vWF, and TAT compared to patients with intermittent claudication. This is consistent with the fact that patients with critical ischaemia also have a significantly higher risk of ischaemic events and mortality compared to claudicants.[3] This is an important finding as previous studies which have assessed hemostatic and inflammatory markers have concentrated mainly on patients with ankle–brachial indexes (ABI) less than 0.9[13,15,18] and data on patients with critical limb ischaemia is limited. Although, there are other markers of both inflammation and coagulation available, selection of D-dimer, TAT, CRP and vWF was based on experience with these markers and previous validation.

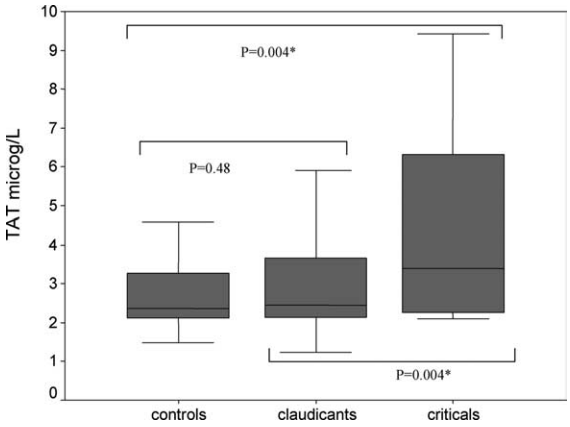

Fig. 4. Boxplots of TAT levels in controls, claudicants and criticals. (Bars = median values; Whiskers = smallest and largest values excluding extremes; top of box + 75th percentile; bottom of box = 25th percentile). *Statistically significant difference.

In contrast to studies involving patients with coronary artery disease there are only a limited number of studies which have assessed CRP levels in patients with peripheral arterial disease. Minor elevations of CRP have been shown to be predictive of cardiovascular events in patients with coronary heart disease.[19] It is now believed that CRP is not merely a marker of low grade chronic systemic inflammation but may be actively involved in atherosclerosis as it can amplify the inflammatory response through complement activation, tissue damage and activation of endothelial cells.[20] Recently, Ridker and colleagues have shown CRP levels to be a strong predictor of future cardiovascular events based on a study of 28,000 women.[21] McDermott and colleagues, have recently shown that CRP was not associated with ankle–brachial pressure index in patients without a history of cardiac or cerebrovascular disease.[18] We have shown that CRP levels are elevated in claudicants and even more so in patients with critical limb ischaemia. It should be known that only 15% of the patients with claudication had a history of known coronary heart disease. The finding of increased CRP levels in claudicants and even more so in patients with critical limb ischaemia, reflects the increased risk of cardiac events and emphasises the need for these patients to receive appropriate secondary risk factor management.[22] We found no difference between males and females in each of the three groups with regards to CRP and vWF levels.

In the Edinburgh Artery Study, elevated plasma levels of vWF were significantly associated with the risk of developing PAD, but not with the progression of the disease.[15] We have shown that patients with claudication had significantly higher levels of vWF than controls and levels were further significantly increased in patients with critical limb ischaemia. Levels of vWF have also been shown to be increased in smokers and patients suffering from hypertension or diabetes where it is considered to reflect endothelial activation.[23–25] In our study, there was no difference in the smoking or hypertensive history in claudicants and patients with critical limb ischaemia. There, were however, more diabetics in the group with critical limb

ischaemia and no diabetics in the control group. Subgroup analysis of patients with claudication and no history of diabetes revealed that vWF were still significantly increased compared to controls. It should also be noted that the three groups of patients were not matched in terms of age due to the need to recruit control patients with no history of coronary artery disease and the fact that critical limb ischaemia is more common in older patients. Levels of vWF have been shown to increase by 0.15 U/ml for each 10 years of age.[26] Allowing for this correction factor, vWF levels were still significantly higher in patients with claudication compared to controls and even higher in criticals. CRP levels also increase with age, but again the differences observed between the groups are very much larger that that which could be attributed to age alone.[27] Furthermore, the difference in both vWF and CRP levels with age may be due to an increased preponderance of vascular disease.

Various markers of coagulation, including the fibrin degradation product D-dimer have been shown to be elevated in patients with peripheral arterial disease. D-dimer levels have also been shown to relate to the severity of the atherosclerosis.[13] McDermott and colleagues have recently shown that D-dimer is inversely associated with the ankle–brachial pressure in patients with and without a history of cardiac or cerebrovascular disease.[18] We have found D-dimer levels to be elevated in claudicants compared to controls and have shown that levels are higher in patients with critical limb ischaemia although this did not reach statistical significance. With regards to the fact that controls were not receiving aspirin in contrast to both patients with claudication and patients with critical ischaemia, D-dimer does not seem to be affected by aspirin treatment.[28,29]

Elevated TAT levels have also been reported in some smaller studies of patients with peripheral arterial disease. However, a recent study of 131 patients showed no correlation between TAT levels and the presence of peripheral arterial disease.[13] We, also found that this marker of thrombin generation was not significantly increased in claudicants but was significantly elevated in patients with critical limb ischaemia. However, administration of aspirin reduces TAT levels[30,31] and, therefore, the fact that patients with claudication and critical ischaemia were receiving aspirin while controls were not, indicates that the difference between patients and controls may have been underestimated.

We have previously shown that platelet activation is significantly increased in patients with peripheral arterial disease compared to controls despite aspirin therapy.[8] Current strategies to reduce the risk of cardiovascular and cerebrovascular events in patients with PAD consist mainly of antiplatelet treatment.[32] If the enhanced levels of coagulation activation observed in patients with peripheral arterial disease play a part in the increased risk in PAD then the implications are that anticoagulants should be added to antiplatelet treatment. Adding anticoagulant drugs to the currently recommended antiplatelet treatment may reduce the risk of ischaemic events observed in this group of patients but would also increase the risk of bleeding. The use of this combined therapy has been advocated in patients undergoing coronary angioplasty.[33,34]

In summary, this study adds to existing evidence for the presence of a hypercoagulable state in patients with peripheral arterial disease. This is even more marked in patients with critical limb ischaemia compared to those with claudication. Whether this increased level of activation is primary or secondary to the atherosclerotic changes in these patients is uncertain. However, the findings that platelet function as well as markers of coagulation and endothelial stimulation are increased in patients with peripheral arterial disease suggest that further research into use of combined antiplatelet and anticoagulation therapy is required in this group of patients.

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