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β2-microglobulin, a novel biomarker of peripheral arterial disease, independently predicts aortic stiffness in these patients

JAAK KALS1,2,3, MAKSIM ZAGURA1,2, MARTIN SERG2,4, PRIIT KAMPUS1,2,4, KERSTI ZILMER1, EVE UNT5,6, JÜRI LIEBREG3,7, JAAN EHA2,4, ANTS PEETSALU7 & MIHHEL ZILMER1,2

1Department of Biochemistry, Centre of Excellence for Translational Medicine, University of Tartu, Tartu, 2Endothelial Centre, University of Tartu, Tartu, 3Department of Vascular Surgery, Tartu University Hospital, Tartu, 4Department of Cardiology, University of Tartu, Tartu, 5Department of Sports Medicine and Rehabilitation, University of Tartu, Tartu, 6Sports Medicine and Rehabilitation Clinic, Tartu University Hospital, Tartu, and 7Department of Surgery, University of Tartu, Tartu, Estonia

Abstract
Arterial stiffness is a prominent feature of vascular ageing and strongly predicts cardiovascular and total mortality. The β2-microglobulin, (β2M) a newly identified biomarker of peripheral arterial disease (PAD), is related to renal insufficiency, inflammatory and neoplastic diseases, but may also play a role in vascular dysfunction. However, the relationship between arterial stiffness and β2M has not been previously studied in patients with atherosclerosis. In the present study we examined a possible association between β2M and arterial stiffness in patients with PAD and in healthy subjects. Plasma β2M levels and parameters of arterial stiffness such as aortic pulse wave velocity (aPWV) and augmentation index (AIx) were measured in 66 patients with PAD and in 66 apparently healthy subjects. Plasma levels of β2M, aPWV and AIx were significantly increased in patients with PAD compared with controls (1858.1 ± 472.8 vs 1554.5 ± 277.9 μm/s, p < 0.001; 9.9 ± 2.2 vs 7.6 ± 1.6 μm/s, p < 0.001; 28 ± 8 vs 14 ± 11%, p < 0.001, respectively). There existed significant correlation between aPWV and β2M for the patient group (R = 0.47; p < 0.001), but not for the controls (R = 0.14; p = 0.26). In multivariate analysis, β2M remained independently associated with aPWV, fetuin-A, age and glomerular filtration rate in patients (R2 = 0.5, p < 0.001). We found no relationship between β2M and AIx in either group. We demonstrated that among patients with PAD elevated plasma β2M levels were associated with higher aortic stiffness irrespective of cardiovascular disease risk factors. These data suggest that β2M may influence the pathogenesis of aortic stiffness in atherosclerosis.

Key Words: Aortic pulse wave velocity, atherosclerosis, β2-microglobulin, peripheral arterial disease

Introduction
Arterial stiffness is a significant manifestation of vascular ageing and a major risk factor for stroke, coronary heart disease, and cardiovascular and total mortality [1–3]. Arterial stiffening reduces the buffering capacity of the main elastic arteries, which leads to increased systolic and pulse pressure, promotes left ventricular hypertrophy and dysfunction, and impairs capacity for myocardial perfusion [4]. Furthermore, increased vascular stiffness becomes a legitimate therapeutic target, particularly in patients at risk of cardiovascular disease [5,6].

Arterial stiffness may also play an important role in atherogenesis [4,7]. Arterial stiffening directly accelerates atheroma formation due to reduction in shear stress, and hence results in decreased production of nitric oxide, which has several anti-atherogenic properties. Arterial stiffness can also lead to remodeling of arterial wall and intima-media thickening. We as well as other researchers have previously demonstrated that increased arterial stiffness occurs in patients with peripheral arterial disease (PAD) [8,9] and, moreover, that increased arterial stiffness influences the functional capacity of these patients [10].

The protein β2-microglobulin (β2M) is a polypeptide, which is one of the major histocompatibility complex class I molecules on the cell surface of all nucleated cells [11]. Free β2M circulates in the blood...
as a result of shedding from cell surfaces, or intracellular release. Increased plasma levels of β2M occur in a variety of autoimmune, neoplastic, infectious and renal diseases [11,12]. Serum β2M level is also a risk factor for carotid artery intima-media thickening [13] and independently predicts total mortality in a general population of older adults [14].

Recently it has been demonstrated that plasma levels of β2M are predictive for PAD and, moreover, that β2M levels independently correlate with disease severity [15]. Thus, the authors suggest that β2M is a potentially specific diagnostic biomarker for PAD. Limited evidence suggests that β2M is associated with development of arterial stiffness [16]. However, relationship between arterial stiffness and β2M has not been previously studied in patients with atherosclerosis. Accordingly, we hypothesized that elevated plasma β2M concentration may be related to arterial stiffening in patients with atherosclerosis. The aim of the current study was to test this hypothesis in patients with PAD, in a model of advanced atherosclerosis, and in apparently healthy subjects.

Methods

Study population

The study group consisted of 66 patients with PAD having stages II, III or IV of chronic ischaemia as defined by Fontaine: stage II – intermittent claudication, stage III – leg pain at rest and stage IV – ulceration or gangrene. All patients were recruited from the Department of Vascular Surgery, Tartu University Hospital, Estonia. The subjects were all male with angiographically proven PAD, i.e. with occlusion of the arteries of the lower extremities. Their ankle brachial index (ABI) was less than 0.9 (range 0.1–0.89). The patients’ exclusion criteria were the following (based on clinical examination, ECG and blood tests): any concomitant acute or chronic inflammatory disease, myocardial infarction, coronary revascularization or cerebrovascular events during the past 6 months, earlier revascularization procedures of the lower limb, upper limb occlusive arterial disease, cardiac arrhythmias, or valve pathologies, diabetes mellitus, malignancies and renal failure. Twenty-one (31.8%) patients with arterial disease, cardiac arrhythmias, or valve pathologies, hypertension (blood pressure ≥ 140/90 mmHg), cerebral or peripheral atherosclerotic disease, diabetes mellitus, malignancies, renal failure and regular use of any medications. This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethics Committee, University of Tartu. Informed written consent was obtained from each participant.

Laboratory methods

Plasma β2M concentration was measured by a chemiluminescent immunoassay using a commercially available kit (L2KBM2, Siemens Medical Solutions Diagnostics®, California, USA) in the IMMULITE 2000 automated analyser (Siemens Medical Solutions Diagnostics®, California, USA). The within-run and total precision coefficients of variation for β2M were 4.2% and 11%, respectively. Blood samples were centrifuged and plasma for fetuin-A was divided into aliquots and stored at −70 °C until analysis. Fetuin-A was measured by an enzyme-linked immunoabsorbent assay using a commercially available kit (BioVendor Laboratory Medicine, Inc.® Brno, Czech Republic). The intra- and inter-assay precision coefficients of variation for fetuin-A were 5.4% and 3.9%, respectively. All determination procedures were performed in accordance with the manufacturer’s recommendations.

Plasma glucose, high-sensitivity C-reactive protein (hsCRP), total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride and creatinine levels were determined by standard laboratory methods using certified assays in the local clinical laboratory. Glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease formula, equation MDRD 1 [17].

Assessment of haemodynamics and arterial stiffness

Peripheral blood pressure was measured in the dominant arm using a validated oscillometric technique (OMRON M4-I; Omron Healthcare Europe BV®,...
Table I. Baseline characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PAD patients (n = 66)</th>
<th>Controls (n = 66)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>63 ± 7.2</td>
<td>54.7 ± 6.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.5 ± 4</td>
<td>26.8 ± 3.4</td>
<td>0.04</td>
</tr>
<tr>
<td>Ankle brachial index</td>
<td>0.42 ± 0.3</td>
<td>1.12 ± 0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.9 ± 1.29</td>
<td>5.2 ± 0.98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.28 ± 0.41</td>
<td>1.35 ± 0.32</td>
<td>0.26</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>4.19 ± 1.18</td>
<td>3.62 ± 0.94</td>
<td>0.003</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.79 ± 0.73</td>
<td>1.06 ± 0.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.61 ± 1.04</td>
<td>5.44 ± 0.49</td>
<td>0.11</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>3.57 (1.2–7.12)</td>
<td>1.09 (0.58–2.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GFR (mL/min/1.73 m²)</td>
<td>102.5 ± 26.5</td>
<td>97.9 ± 18.2</td>
<td>0.25</td>
</tr>
<tr>
<td>β2M (μg/L)</td>
<td>1858.1 ± 472.8</td>
<td>1554.5 ± 277.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fetuin-A (μg/mL)</td>
<td>260.2 ± 106</td>
<td>254.8 ± 104.9</td>
<td>0.77</td>
</tr>
<tr>
<td>AIX (%)</td>
<td>28 ± 8</td>
<td>14 ± 11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aPWV (m/s)</td>
<td>9.9 ± 2.2</td>
<td>7.6 ± 1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>64 (97)</td>
<td>2 (3)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

The values are expressed as means (± SD), medians (with 25% and 75% percentiles) or prevalence (%). BMI, body mass index; hsCRP, high-sensitivity C-reactive protein; AIX, augmentation index; GFR, glomerular filtration rate; β2M, β2-microglobulin; aPWV, aortic pulse wave velocity.
(134 ± 19 mmHg vs 118 ± 17 mmHg, p < 0.001) were also different for the patients and for the controls. Differences between the groups occurred also in medication use: 25 (37.9%) patients were on pentoxyfylline treatment, 14 (21.2%) patients received aspirin, 23 (34.8%) patients received antihypertensive drugs and 6 (9.1%) were on statin therapy, while the controls did not receive chronically any medications.

Relationship between aortic stiffness and other variables

Linear regression analysis was used to establish whether aPWV correlated with other variables within each group separately. A significant relationship was found between aPWV and GFR or age for the patient (R = −0.34, p = 0.005; R = 0.42, p < 0.001, respectively) and for the control groups (R = −0.26, p = 0.03; R = 0.53, p < 0.001, respectively). There was a significant positive association between aPWV and β2M only for the patients (Figure 1). At the same time we found no relationship between aPWV and β2M for the control group, or between AIx and β2M for either group (data not shown). To determine whether the association between aPWV and β2M was independent of traditional cardiovascular risk factors or specific factors influencing arterial stiffness, a multiple linear regression model was developed with aPWV as the dependent variable for each group separately. Aortic PWV was independently associated only with β2M and age for the patient group (Table II), whereas there occurred a significant independent correlation of aPWV with MAP and age in the healthy subjects (R² = 0.41, p < 0.001).

Relationship between β2-microglobulin and other variables

A significant linear relationship was observed between β2M and GFR or age for the patient (R = −0.45, p < 0.001; R = 0.48, p < 0.001, respectively) and for the control groups (R = −0.43, p < 0.001; R = 0.31, p = 0.01, respectively), but there was a significant correlation between β2M and fetuin-A level only for the patient group (Figure 2). To determine which variables were independently associated with β2M, a multiple linear regression model was developed for each group with β2M as the dependent variable. The final models indicated that β2M was positively associated with fetuin-A levels, aPWV, age and GFR for the patient group (Table III), while in the healthy subjects β2M was significantly positively correlated with age and GFR (R² = 0.24, p < 0.001).

Discussion

The current study is the first clinical analysis to examine the relationship between aortic stiffness and β2M in patients with PAD and in healthy controls. Our main novel finding was that aPWV, ‘gold-standard’ measure of arterial stiffness, is determined independently by β2M levels in patients with advanced atherosclerosis. Arterial stiffness, which can be measured noninvasively (e.g. aPWV), is a strong predictor for cardiovascular outcome and is known to be an indicator and mediator of development of atherosclerotic disease. Our finding about the independent association between β2M level and aortic stiffness in patients with PAD suggests that β2M may be related to the pathological stiffening of the aorta in advanced atherosclerosis.

Because PAD is largely underdiagnosed and undertreated, these individuals are at high risk of myocardial infarction, stroke and cardiovascular death [18]. Therefore, early diagnosis and assessment of cardiovascular risk in these patients is a significant clinical challenge. Measurement of ABI provides a relatively simple and non-invasive diagnostic test that has very high sensitivity and specificity, when the test is performed by a trained individual [19]. However, the primary practitioner seldom performs ABI in an office setting. Recognition of PAD would trigger intensive risk factor modification and therapy even in the patient without classic symptoms. Discovery of soluble biomarkers and non-invasive imaging technologies that are highly associated with subclinical atherosclerosis would greatly aid in identifying patients with PAD [20,21]. Recently, Wilson with co-authors, by using extensive proteomic profiling, identified plasma levels of β2M as a potentially diagnostic biomarker of PAD [15]. Moreover, β2M levels independently correlated with severity of the disease as assessed by ABI or treadmill testing [15].
In view of the above findings, we examined plasma levels of β2M and functional indices of aortic stiffness and pulse wave reflection to determine the possible contribution of the new biomarker β2M to arterial stiffness in PAD patients. We found increased plasma levels of β2M and indices of arterial stiffness (aPWV, AIx) in the patients compared with the controls (Table I). Previous studies have also demonstrated higher aPWV and AIx in patients with PAD [8,9] as well as an association between increased vascular stiffness and reduced arterial flow in the lower extremities [22]. Brachial-ankle PWV and AIx predict walking ability in these patients [10,23], moreover, improvement in arterial stiffness enhances walking ability in patients with PAD [24]. These data point to the close association of arterial stiffness with lower extremity atherosclerosis, which could be an important explanation for linking PAD with reduced walking ability in patients with PAD [24].

Our results confirm previous preliminary data [15,25,26] about β2M plasma levels being elevated in PAD patients compared with healthy subjects. In this study, we demonstrated that aPWV was significantly independently determined by β2M levels and age only for the patient group (Figure 1, Table II). To the best of our knowledge, this is the first study to clarify the significant association between β2M and aPWV in patients with lower extremity atherosclerosis. Previously, Saijo and coauthors demonstrated association between β2M and arterial stiffness in subjects from the general population [16]. However, they measured brachial-ankle PWV using the volume-plethysmographic technique. We assessed aortic stiffness by carotid-femoral PWV which is the current ‘gold-standard’ measure of arterial stiffness [5]. Moreover, aPWV represents subclinical organ damage and is considered a valid surrogate end-point that can be used in intervention studies [5,6]. Our results suggest that plasma β2M level may be a potentially specific marker of PAD and associated with pathological stiffening of the aorta and the large arteries, irrespective of the effects of potential confounders (e.g. distending pressure, dyslipidemia, hyperglycaemia, renal function and inflammation). Interestingly, we found no association of aPWV or β2M with ABI for either group (data not shown), which can probably be attributed to small sample size.

We believe that elevation of β2M in PAD patients is probably multifactorial, reflecting the effects of renal function, ischaemia-reperfusion injury and vascular inflammation. In patients with renal insufficiency β2M levels are elevated and associated with inflammatory response during haemodialysis [27]. Previously, it has also been hypothesized that β2M could be released to the systemic circulation from ischaemic muscle as a result of ischaemia-reperfusion injury, which typically occurs during exercise in patients with PAD. However, interestingly, a recent study by Busti and co-authors demonstrated that acute ischaemia-reperfusion damage produced by maximal treadmill exercise did not enhance plasma β2M in patients with PAD [25].

The exact mechanism whereby elevated level of β2M could cause large artery stiffening remains thoroughly unclear. There is growing evidence that inflammation plays a prominent role in atherosclerotic syndromes. Thus, as β2M has an impact on immunity and inflammation, the association between

### Table II. Multiple regression model for patients with aPWV as the dependent variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>Standard error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2M (μg/L)</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.084</td>
<td>0.038</td>
<td>0.031</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>0.025</td>
<td>0.018</td>
<td>0.168</td>
</tr>
</tbody>
</table>

$R^2 = 0.3, p < 0.001$. β2M, β2-microglobulin; MAP, mean arterial pressure.

### Table III. Multiple regression model for patients with β2M as the dependent variable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression coefficient</th>
<th>Standard error</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin-A (μg/mL)</td>
<td>1.655</td>
<td>0.409</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aPWV (m/s)</td>
<td>51.729</td>
<td>21.485</td>
<td>0.019</td>
</tr>
<tr>
<td>Age (years)</td>
<td>16.496</td>
<td>6.883</td>
<td>0.02</td>
</tr>
<tr>
<td>GFR (mL/min/1.73 m²)</td>
<td>−3.772</td>
<td>1.809</td>
<td>0.041</td>
</tr>
</tbody>
</table>

$R^2 = 0.5, p < 0.001$. β2M, β2-microglobulin; aPWV, aortic pulse wave velocity; GFR, glomerular filtration rate.
β2M and aPWV in patients with atherosclerosis could be related to vascular inflammation [28]. Surprisingly, we found no association of β2M and aPWV with the inflammatory markers (hsCRP, WBC, data not shown). Yet there occurred a significant association of β2M and aPWV with GFR for both groups, and a significant independent correlation between β2M and fetuin-A levels for the patient group (Figure 2, Table III). Previously it has been shown that there are links between renal function and β2M [12] and arterial stiffness [29], which points to the possible role of β2M in renal-induced vascular stiffening. As some authors have recently established independent correlation between the levels of the calcification inhibitor fetuin-A and arterial stiffness [30], we hypothesized that β2M-associated aortic stiffening could be related, at least in part, to vascular calcification. Alternatively, it is possible that β2M may damage vessel walls by participating in amyloid formation in the arteries [27].

Limitations

The weaknesses of the present study are its cross-sectional observational nature as well as the relatively small sample size. Considering the design of the present study, the causality of the biochemical mechanisms responsible for alterations in the vascular wall remains speculative. As such, these data provide primarily mechanistic insights into the relative contribution of β2M to aortic stiffness.

An additional limitation of the study is the potential confounding long-term effects of medications and smoking. Therefore, a potential bias should be considered. However, to minimize the effect of different drugs and smoking on the results, medication and smoking were discontinued at least 12 h before the study. Finally, considering the age composition of our sample and the fact that PAD patients are predominantly men, the extrapolation of our findings to younger individuals and females is questionable.

Conclusions

Our data demonstrate that, in PAD patients, elevated plasma β2M levels were associated with higher aortic stiffness irrespective of known atherogenic risk factors. These data suggest that β2M may play an important role in aortic stiffening in atherosclerosis. Moreover, our findings indicate that combined assessment of aPWV and β2M may provide useful functional and biochemical information about vascular risk in the setting of PAD. Further investigations are needed to elucidate the exact mechanisms of β2M in the pathogenesis of arterial stiffening, as well as to assess whether reducing β2M levels improves parameters of arterial elasticity.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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