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## A biomarker panel for peripheral arterial disease

Eric T Fung<sup>1</sup>, Andrew M Wilson<sup>2</sup>, Fujun Zhang<sup>1</sup>, Nathan Harris<sup>1</sup>, Kim A Edwards<sup>2</sup>, Jeffrey W Olin<sup>3</sup> and John P Cooke<sup>2</sup>

**Abstract:** Peripheral arterial disease (PAD) is common, but often not diagnosed. A biomarker index would be useful to raise suspicion of PAD, so as to trigger appropriate vascular testing and management. The study comprised 540 individuals: 197 individuals with both coronary artery disease and peripheral arterial disease (CAD + PAD); 81 with CAD only; and 262 with no hemodynamically significant disease (NHSD) of the coronary or peripheral arteries. Multiple linear regression was performed to generate a biomarker panel score that could predict ankle-brachial index (ABI). Logistic regression was used to investigate the relationship between disease status and the panel score as well as other risk factors (e.g. age, diabetes status, smoking status). ROC analysis was performed to test the prediction power of the biomarker panel score. Among the plasma markers tested, beta 2 microglobulin ( $\beta$ 2M) and cystatin C had the highest correlation with ABI, and higher than any of the conventional risk factors of age, smoking status, and diabetes status. A biomarker panel score derived from  $\beta$ 2M, cystatin C, hsCRP, and glucose had an increased association with PAD status (OR = 12.4, 95% confidence interval (CI) 6.6–23.5 for highest vs lowest quartile), which was still significant after adjusting for known risk factors (OR = 7.3, 95% CI 3.6–14.9 for highest vs lowest quartile). In conclusion, after taking into account the traditional risk factors for PAD, a biomarker panel comprising  $\beta$ 2M, cystatin C, hsCRP, and glucose adds useful information to assess the risk of disease.

**Key words:** beta 2 microglobulin; C-reactive peptide; cystatin C; glucose; proteomics

### Introduction

Peripheral arterial disease (PAD) affects 8 to 12 million individuals in the United States and is also prevalent in Europe and Asia.<sup>1–4</sup> Classically, PAD causes limb fatigue or pain brought on by exertion and is relieved by rest (i.e. intermittent claudication), and reduces functional capacity and quality of life.<sup>5,6</sup> It is frequently associated with coronary and cerebral disease.<sup>6</sup> Patients with PAD are at increased risk from myocardial infarction, stroke, aortic aneurysm, and vascular death, as well as ischemic ulceration and amputation.<sup>7</sup>

The high risk of vascular events in PAD is reduced by aggressive risk factor modification. In these individuals, the use of statins, angiotensin converting enzyme inhibitors, and antiplatelet therapy

reduces morbidity and mortality.<sup>8</sup> Unfortunately, PAD is underdiagnosed and undertreated. In fact, many of those affected do not manifest the classic symptomatology. Classic claudication is only noted by 10–30% of patients<sup>6,9</sup> and atypical leg discomfort occurs in 20–40%.<sup>6</sup> Up to 50% of patients do not complain of leg symptoms. However, even these individuals have a reduced lifespan without aggressive treatment.<sup>10,11</sup> Thus, it is important to diagnose PAD, even in the patient without classic symptomatology. Discovery of biomarkers that are highly associated with PAD would aid greatly in identifying such patients.

Biomarker index scores are increasingly used in medicine to refine diagnosis and to aid in prognostication. For example, such index scores are used to assess the risk of progressing from cirrhosis to hepatocellular carcinoma in patients infected with hepatitis virus,<sup>12</sup> or to assess the likelihood that breast cancer will recur.<sup>13</sup> Generally, these index scores perform better than individual markers. Few studies have explored combinations of markers to create a discrete index score to stratify individuals according to their risk of having PAD. None have used an agnostic proteomic profiling approach to develop a biomarker index. We have done so, and reported that beta 2 microglobulin ( $\beta$ 2M) and cystatin C were preferentially elevated in patients with

<sup>1</sup>Vermillion, Inc., Fremont, CA, USA; <sup>2</sup>Division of Cardiovascular Medicine, Stanford University, Stanford, CA, USA; <sup>3</sup>Zena and Michael A Wiener Cardiovascular Institute, Mount Sinai Medical Center, New York, NY, USA

Correspondence to: John P Cooke, Stanford University School of Medicine Division of Cardiovascular Medicine, 300 Pasteur Drive, Falk Cardiovascular Research Center, Stanford, CA 94305-5406, USA. Email: john.cooke@stanford.edu

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PAD.<sup>14</sup> The current study extends that work to develop and validate a PAD biomarker panel.

## Methods

### Individuals

A total of 540 individuals were investigated in the study. They were randomly selected from the ongoing GenePAD study.<sup>15</sup> Of these 540 patients, 44% are a subset of the patient sample described in Wilson, *et al.*<sup>14</sup> However, in the current study, we used a fresh aliquot of plasma for all patients, and measurements of  $\beta$ 2M and cystatin C were performed *de novo*.

This study of the genetic determinants of PAD comprises individuals undergoing coronary angiography at Stanford University or Mount Sinai Medical Centers. The PAD status of these individuals was not known to the investigators at the time of informed consent and recruitment into the study. Ankle-brachial index (ABI) was determined immediately after recruitment, followed by a comprehensive clinical characterization which included coronary angiography. Patients with PAD had a resting ABI of  $< 0.90$  or, in those with non-compressible ankle arteries, a toe-brachial index of  $< 0.60$ . The glomerular filtration rate (GFR) was estimated by the Modification of Diet in Renal Disease (MDRD) method.<sup>16</sup>

Coronary angiograms were reviewed by an experienced angiographer blinded to the individual's ABI. A significant coronary lesion was defined as an angiographic stenosis of  $\geq 60\%$  in any vessel. The GenePAD study was funded by the National Heart, Lung and Blood Institute (NHLBI) and approved by the Stanford University and Mount Sinai School of Medicine Committees for the Protection of Human Subjects.

### Measurement of markers

Venipuncture was performed on fasting individuals and serum and plasma samples were stored at  $-75^{\circ}\text{C}$ . Glucose, high-density lipoprotein cholesterol (HDL), triglycerides and total cholesterol were all measured by standard assays using the AU5400 Chemistry Immuno-Analyzer (Olympus Inc). Low-density lipoprotein cholesterol (LDL) was measured by standard assay using the AU640 Chemistry Immuno-Analyzer (Olympus). Beta 2 microglobulin, high-sensitivity C-reactive protein (hsCRP) and cystatin C assays were measured using standard nephelometry using the BNII-Nephelometry system (Dade Behring Inc). All assay reagents were supplied by the relevant manufacturer with the exception of the  $\beta$ 2M nephelometric kit (The Binding Site, Inc). Controls were pur-

chased from Bio-Rad Laboratories or Cliniqua Corporation.

### Data analysis and statistics

Dichotomous variables are expressed as prevalence in number and percent, and continuous data are expressed as the median (25th to 75th percentiles). Univariate comparisons of risk factors and laboratory results were calculated using the Fisher exact test for dichotomous variables and using the non-parametric Mann-Whitney *U*-test for continuous variables. Spearman coefficient of rank correlation was performed to assess the relationship of data to ABI. Calculations were performed using Prism 4.0 (Graphpad). Multiple linear regression was performed using all combinations of the markers to generate a multi-marker panel score that could predict ABI. The biomarker values were treated as continuous variables. The score was derived from the equation:  $1.169069 + (0.020299 * \beta 2M) - (0.004312 * \text{hsCRP}) - (0.149757 * \text{cystatin C}) - (0.000976 * \text{glucose})$ . Because the output of the linear regression analysis was positively correlated with ABI, the biomarker panel score was defined as the inverse of the linear regression output so that a higher score would indicate a higher risk. Logistic regression was used to investigate the relationship between the disease status and the biomarker panel score as well as other risk factors (e.g. age, diabetes status, smoking status). Receiver operating characteristic (ROC) analysis<sup>17</sup> was performed to test the predictive power of the biomarker panel score. All individuals were assigned a score using the American Heart Association (AHA) Framingham risk score charts based on data obtained at recruitment.

The odds ratio was calculated in the logistic regression analysis. *R* was used in the linear regression analysis. SAS was used for logistic regression analysis and odds ratio calculation. Analyze-it was used for ROC analysis.

## Results

All study participants underwent coronary angiography ( $n = 540$ ), and included a group with no hemodynamically significant atherosclerosis (NHSD;  $n = 262$ ); one with CAD + PAD ( $n = 197$ ); and a group with CAD alone ( $n = 81$ ). The CAD + PAD group was slightly older than the NHSD group, and was more likely to be receiving medications for hypertension (86% vs 71%) for hypercholesterolemia (79% vs 56%), and for diabetes (45% vs 20%). All biochemical markers for cardiovascular risk were higher in the CAD + PAD group than in the NHSD group (Table 1).

**Table 1** Participant demographics and biomarkers

	NHSD ( <i>n</i> = 262)	CAD/PAD ( <i>n</i> = 197)	CAD ( <i>n</i> = 81)	NHSD vs CAD/PAD	CAD/PAD vs CAD
Male sex, <i>n</i> (%)	138 (53%)	77 (39%)	43 (46%)	0.005	0.23
Age, years	63 (56–71)	70 (64–77)	72 (67–78)	< 0.001	0.14
Body mass index, kg/m <sup>2</sup>	28.3 (24.7–33.1)	27.9 (25.1–31.6)	28.5 (25.9–31.8)	0.3	0.32
Smoking, <i>n</i> (%)	119 (45%)	135 (69%)	45 (56%)	< 0.001	0.05
Hypertension, <i>n</i> (%)	185 (71%)	170 (86%)	70 (86%)	< 0.001	1.0
Diabetes, <i>n</i> (%)	52 (20%)	89 (45%)	22 (27%)	< 0.001	0.007
Resting ABI	1.11 (1.02–1.19)	0.79 (0.7–0.9)	1.07 (1.0–1.14)	< 0.001	< 0.001
GFR, ml/min	86.3 (63.1–108)	64.3 (46.7–84.6)	71.4 (50.2–94.6)	< 0.001	0.16
β2M, mg/dl	1.67 (1.41–2.05)	2.19 (1.7–3.4)	1.92 (1.57–2.43)	< 0.001	0.002
Cystatin C, mg/dl	0.66 (0.58–0.77)	0.83 (0.7–1.2)	0.77 (0.65–0.89)	< 0.001	0.016
hsCRP, mg/l	1.5 (0.6–3.7)	2.2 (0.9–6.3)	1.4 (0.7–3.9)	< 0.001	0.031
Triglycerides, mg/dl	88 (63–131)	105 (70.2–145)	91.1 (68.9–130)	0.007	0.166
Total cholesterol, mg/dl	150 (125–173)	138 (110–157)	132 (107–152)	< 0.001	0.184
LDL cholesterol, mg/dl	85 (66–109)	73 (58–95)	77 (58–94)	< 0.001	0.808
HDL cholesterol, mg/dl	42 (34–51)	38 (30–45)	37 (30–46)	0.003	0.91
Glucose, mg/dl	85.4 (79.2–96.1)	94.6 (82.7–130)	91.1 (80.8–102)	< 0.001	0.085

Abbreviations defined in text.

Correlation analysis was performed to determine which characteristics were most highly associated with ABI. The traditional risk factors most strongly associated with PAD were diabetes status and age (Table 2). Among biochemical markers, β2M and cystatin C correlated most strongly with ABI.

Linear regression using these variables was performed to generate indices for various combinations of two, three, and all four biomarkers. For the four marker index, each of the biomarkers is significant ( $p < 0.001$ ) in the model. These linear regression indices were positively correlated with ABI, i.e. a lower value of the linear regression index indicated lower ABI. The biomarker panel score was defined

as the inverse of the linear regression index so that a higher biomarker panel score would indicate higher likelihood of positive PAD status. The odds ratio was calculated for each of the quartiles for each of the individual markers as well as for each of the combinations (Table 3). The panel score comprising all four markers had the highest odds ratio when comparing the highest quartile versus the lowest quartile, and its significance was still apparent even after adjusting for traditional risk factors of age, diabetes, and smoking. The quartile cutoffs for the marker combination are quartile 1, 0.949651; median, 0.994995; and quartile 3, 1.015032. After adjusting for the traditional risk factors, individuals in the top quartile of the four marker index had a sevenfold greater chance of having PAD (Table 3). We performed the Hosmer and Lemeshow Goodness-of-Fit Test, with the resulting chi-square of  $< 0.0001$  and  $p > 0.9999$ , indicating there was not a lack of fit of the model. In addition, each of the terms in the regression model was statistically significant ( $p < 10^{-4}$ ).

ROC analysis was performed to determine the diagnostic accuracy of conventional risk factors, the AHA score, and the biomarker panel (Figure 1). ROC analysis of conventional risk factors (age, diabetes, and smoking) resulted in an AUC of 0.78 (95% confidence interval (CI) 0.73–0.82), and addition of the biomarker score to these factors increased the AUC to 0.82 (95% CI 0.78–0.85), which was statistically significant ( $p = 0.014$ ). ROC analysis using the AHA score in the prediction of PAD resulted in an AUC of 0.70 (95% CI 0.65–0.75), and addition of the biomarker score increased the AUC to 0.76 (95% CI 0.72–0.81),  $p = 0.0002$ .

**Table 2** Spearman correlations between risk factors, biomarkers and ABI

	<i>r</i>	95% CI	<i>p</i>
Sex	-0.017	-0.102, 0.069	0.69
Age	-0.231	-0.310, -0.148	< 0.001
Body mass index, kg/m <sup>2</sup>	0.017	-0.069, 0.102	0.694
Smoking	-0.159	-0.242, -0.075	< 0.001
Hypertension	-0.148	-0.230, -0.063	< 0.001
Diabetes	-0.239	-0.318, -0.157	< 0.001
GFR	0.238	0.155, 0.318	< 0.001
β2M, mg/l	-0.297	-0.373, -0.217	< 0.001
Cystatin C, mg/l	-0.302	-0.378, -0.222	< 0.001
hsCRP, mg/l	-0.180	-0.261, -0.096	< 0.001
Triglycerides, mg/dl	-0.110	-0.194, -0.025	0.009
Total cholesterol, mg/dl	0.031	-0.055, 0.116	0.472
LDL, mg/dl	0.092	0.006, 0.176	0.031
HDL, mg/dl	0.001	-0.084, 0.087	0.97
Glucose, mg/dl	-0.200	-0.281, -0.116	< 0.001

Abbreviations defined in text.

**Table 3** Unadjusted and adjusted risks for the diagnosis for PAD based on biomarkers

Marker	Model 1	Model 2	Model 3	Model 4	Model 5
$\beta$ 2M	6.1 (3.0–12.2)	3.2 (1.5–6.9)	5.7 (2.8–11.6)	5.4 (2.6–11.0)	2.5 (1.1–5.5)
Cystatin C	5.6 (2.9–10.8)	3.1 (1.5–6.2)	5.3 (2.7–10.3)	5.3 (2.7–10.3)	2.6 (1.3–5.4)
hsCRP	2.2 (1.2–3.8)	2.8 (1.5–5.3)	2.3 (1.3–4.0)	1.9 (1.0–3.3)	2.6 (1.3–4.9)
Glucose	3.4 (1.9–6.0)	1.5 (0.8–2.7)	2.3 (1.2–4.2)	3.3 (1.8–5.8)	1.8 (0.9–3.5)
$\beta$ 2M + hsCRP	3.2 (1.9–5.6)	4.1 (2.3–7.5)	3.1 (1.8–5.4)	2.9 (1.6–5.0)	3.6 (1.9–6.8)
$\beta$ 2M + cystatin C	5.9 (3.3–10.4)	4.1 (2.3–7.5)	5.6 (3.1–10.0)	5.6 (3.1–10.0)	3.5 (1.9–6.7)
$\beta$ 2M + glucose	5.7 (3.2–10.1)	5.7 (3.1–10.5)	3.9 (2.1–7.2)	5.5 (3.1–10.0)	3.6 (1.8–7.1)
hsCRP + cystatin C	6.9 (3.8–12.4)	6.7 (3.6–12.6)	6.6 (3.6–12.2)	6.2 (3.3–11.4)	6.1 (3.1–12.0)
hsCRP + glucose	5.9 (3.3–10.4)	6.3 (3.4–11.5)	4.3 (2.4–7.9)	5.5 (3.0–9.9)	4.1 (2.1–7.9)
Cystatin C + glucose	6.8 (3.8–12.1)	6.3 (3.4–11.6)	4.9 (2.6–9.1)	6.4 (3.5–11.6)	3.9 (2.0–7.8)
$\beta$ 2M + hsCRP + cystatin C	6.8 (3.8–12.1)	5.2 (2.8–9.5)	6.7 (3.7–12.2)	6.1 (3.4–11.1)	4.6 (2.4–8.8)
$\beta$ 2M + hsCRP + glucose	5.6 (3.2–9.9)	6.4 (3.5–11.8)	4.1 (2.3–7.5)	5.2 (2.9–9.3)	4.2 (2.2–8.3)
hsCRP + cystatin C + glucose	6.8 (3.8–12.1)	6.9 (3.7–12.8)	5.0 (2.7–9.3)	6.2 (3.4–11.3)	4.5 (2.3–8.8)
$\beta$ 2M + cystatin C + glucose	10.8 (5.9–20.1)	8.4 (4.4–15.9)	8.4 (4.4–16.0)	10.0 (5.4–18.9)	5.4 (2.7–10.8)
$\beta$ 2M + hsCRP + cystatin C + glucose	12.4 (6.6–23.5)	11.7 (6.0–22.6)	9.4 (4.8–18.2)	11.2 (5.9–21.4)	7.3 (3.6–14.9)

Data expressed as odds ratio for highest quartile versus lowest quartile (95% confidence interval). See Appendix for calculation of quartiles.

Abbreviations defined in text.

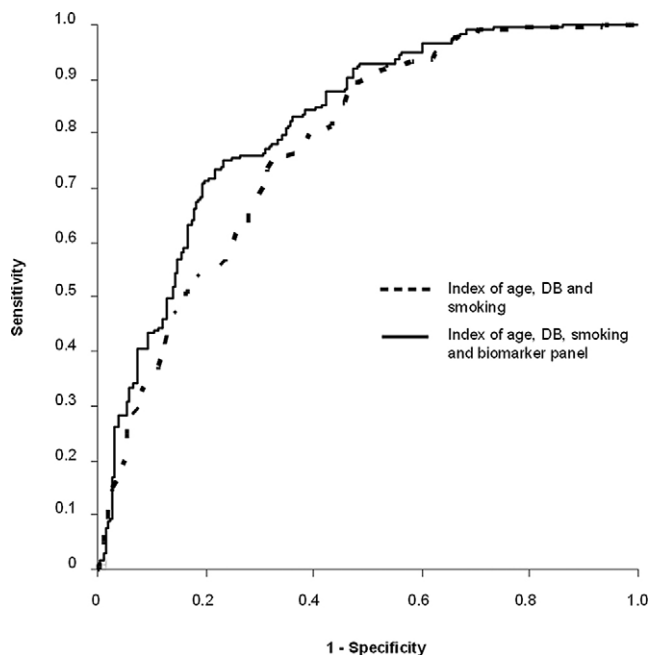
Model 1, unadjusted; Model 2, adjusted for age; Model 3, adjusted for diabetes status; Model 4, adjusted for smoking; Model 5, adjusted for age, diabetes, smoking.

For all models,  $p < 0.05$ .

Because these results compare patients with CAD and PAD with those with no significant hemodynamic disease, the elevation in biomarkers might be associated with the pathophysiology of CAD, of PAD, or both. Thus, the series of individuals with only CAD ( $n = 81$ ) was compared with the CAD +

PAD group. These groups were very similar with respect to the burden of the traditional risk factors, although diabetes and tobacco use were more prevalent in the CAD + PAD group. The mean value of each of the biomarkers – cystatin C, CRP and  $\beta$ 2M – was greater in the CAD + PAD group (Table 1). The biomarker panel score was able to distinguish between the CAD group and the CAD + PAD group, although the AUC of the ROC was lower than that obtained when comparing the CAD + PAD versus NHSD groups (Table 4).

Figure 2 illustrates the odds ratio of CAD + PAD status in analyses in which study participants were stratified into nine groups according to the value of the AHA risk score and that of the four biomarker panel score. Individuals were assigned an AHA risk score using the traditional cardiovascular risk factors as described.<sup>18</sup> AHA risk scores of  $< 5$  (low), 5–10 (medium), and  $> 10$  (high) were associated with increasing risk of PAD ( $p = 0.006$  for men and  $< 0.001$  for women using the score from the linear regression by ANOVA). The odds ratio was calculated by comparing each of the eight groups to the one with the lowest risk of disease (low AHA risk score and low biomarker panel score). Individuals with a low AHA risk score and a low biomarker panel score had the least risk of PAD. Individuals with a high AHA risk score and a high biomarker panel score had the greatest risk of PAD. There was a trend towards increasing risk of PAD as a function of increased AHA risk in combination with an increased PAD panel score.



**Figure 1** ROC analysis of conventional risk factors, the biomarker panel score, and a combination of conven-

**Table 4** Area under the curve derived from receiver-operator curves for the diagnosis of PAD using combination biomarkers

Marker	NHSD vs CAD + PAD	CAD vs CAD + PAD
$\beta$ 2M	0.697 (0.648, 0.746)	0.613 (0.544, 0.681)
Cystatin C	0.704 (0.655, 0.752)	0.593 (0.524, 0.662)
hsCRP	0.593 (0.54, 0.645)	0.583 (0.511, 0.655)
Glucose	0.637 (0.585, 0.69)	0.563 (0.492, 0.633)
$\beta$ 2M + hsCRP	0.617 (0.565, 0.668)	0.600 (0.529, 0.671)
$\beta$ 2M + cystatin C	0.690 (0.641, 0.74)	0.557 (0.486, 0.627)
$\beta$ 2M + glucose	0.677 (0.627, 0.726)	0.606 (0.536, 0.675)
hsCRP + cystatin C	0.669 (0.62, 0.718)	0.612 (0.542, 0.682)
hsCRP + glucose	0.683 (0.632, 0.733)	0.627 (0.559, 0.696)
Cystatin C + glucose	0.709 (0.66, 0.757)	0.623 (0.554, 0.691)
$\beta$ 2M + hsCRP + cystatin C	0.693 (0.644, 0.741)	0.589 (0.52, 0.659)
$\beta$ 2M + hsCRP + glucose	0.691 (0.642, 0.741)	0.639 (0.571, 0.707)
$\beta$ 2M + cystatin C + glucose	0.734 (0.687, 0.781)	0.608 (0.539, 0.676)
hsCRP + cystatin C + glucose	0.719 (0.671, 0.766)	0.65 (0.583, 0.717)
$\beta$ 2M + hsCRP + cystatin C + glucose	0.747 (0.702, 0.791)	0.636 (0.568, 0.703)

Abbreviations defined in text.

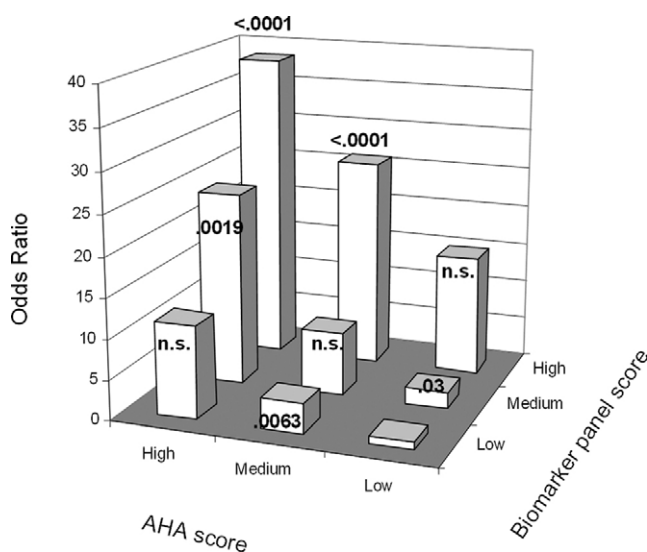
## Discussion

In this study, we demonstrated that a four biomarker panel comprising  $\beta$ 2M, cystatin C, hsCRP, and glucose is associated with the presence of PAD independent of traditional risk factors. The biomar-

kers  $\beta$ 2M and cystatin C were chosen based upon a comprehensive proteomic profiling effort that included over 1600 plasma protein peaks by mass spectroscopy.<sup>14</sup> Of the 1600 peaks, those representing  $\beta$ 2M and cystatin C were most strongly correlated with the ABI.<sup>14</sup> Thus, these biomarkers were chosen from a much larger pool of possible biomarker candidates. The current manuscript describes a logical extension of that work, now showing that individuals in the top quartile of the four biomarker panel score had a sevenfold greater risk of PAD. Thus, we were able to identify individuals at a greatly increased risk for the presence of PAD, even after adjusting for traditional CV risk factors. Such a biomarker panel may be useful in alerting the clinician to the possibility of PAD in patients who might otherwise be undiagnosed.

These findings extend our recent observations using plasma proteomic profiling for PAD. We found that  $\beta$ 2M and cystatin C are associated with PAD and systemic atherosclerosis.<sup>14</sup> Several other biomarkers have been described as having an increased association with PAD including CRP,<sup>19,20</sup> pregnancy-associated plasma protein A,<sup>21</sup> lipoprotein(a),<sup>22,23</sup> interleukins, and fibrinogen.<sup>24</sup> However, these markers were not studied in models that incorporated other risk factors and/or biomarkers.

Cystatin C was included in our model as it was implicated as a biomarker of PAD by our initial proteomic profiling studies,<sup>14</sup> and by a previous report.<sup>25</sup> Several studies have shown that cystatin C is more strongly associated with all-cause and cardiovascular mortality than other measures of renal function such as serum creatinine or estimated GFR,<sup>25–28</sup> underlining the strong link between impaired renal function and atherosclerosis. Like cystatin C,  $\beta$ 2M is associated with renal dysfunction.<sup>29,30</sup> In patients on dialysis,  $\beta$ 2M levels



**Figure 2** Odds ratio of CAD + PAD status by AHA risk score and by biomarker panel score. AHA risk score using the traditional cardiovascular risk factors as described<sup>18</sup> of < 5 (low), 5–10 (medium) and > 10 (high) were associated with increasing risk of PAD ( $P=0.006$  for men and  $P<0.001$  for women using the score from the linear regression by ANOVA). The tertile cutoffs of the biomarker panel score were used to determine the risk level: low (< 0.991), medium (0.991–1.033), and high (> 1.033). The  $n$  in each group were as follows: low AHA/low panel score,  $n=8$ ; low AHA/medium panel score,  $n=17$ ; low AHA/high panel score,  $n=32$ ; medium AHA/low panel score,  $n=71$ ; medium AHA/medium panel score,  $n=81$ ; medium AHA/high panel score,  $n=75$ ; high AHA/low panel score,  $n=57$ ; high AHA/medium panel score,  $n=39$ ; high AHA/high panel score,  $n=30$ . ns, not significant.

are greatly elevated and contribute to amyloid deposition with associated cardiovascular dysfunction.<sup>31</sup> Elevated levels of  $\beta$ 2M have been associated with atherosclerosis<sup>32</sup> but not with PAD until our recent report.<sup>14</sup> The association of cystatin C and  $\beta$ 2M with PAD may in part reflect the association between renal insufficiency and PAD, which has been demonstrated in several studies  $\beta$ 2M.<sup>33,34</sup> However, we have previously shown that the relationship between  $\beta$ 2M and PAD was largely independent of estimated creatinine clearance.<sup>14</sup>

Another explanation for the elevation of  $\beta$ 2M in PAD is the fact that this protein may be shed from cells with injury or inflammation. The original hypothesis driving our proteomic profiling effort was that in PAD patients a repetitive ischemia-reperfusion injury of the limbs might cause characteristic changes in the microvasculature and tissue, including the shedding of endothelial proteins.<sup>20</sup> Notably,  $\beta$ 2M is not covalently attached to the major histocompatibility complex, which may explain its tendency to be released during injury.

Inflammation plays a prominent role in atherosclerotic syndromes.<sup>20</sup> Because of its probable role in immunity and inflammation, the association of  $\beta$ 2M with PAD or with alterations in vascular structure<sup>35</sup> could also be related to vascular inflammation.<sup>36</sup> The inflammatory modulator CRP is also increased in patients with atherosclerosis and is predictive of the development of PAD.<sup>37</sup>

We hypothesized that combining biomarkers may create a panel with higher classification accuracy than the individual biomarkers, in part because each marker may reflect different pathophysiologies contributing to PAD. CRP levels did not correlate strongly with renal function in our study (data not shown). In fact, CRP levels have been shown to be significantly related to adiposity and insulin resistance in a range of population studies, whereas renal impairment is often associated with reduced body mass, particularly in elderly individuals. Thus, we suggest that the use of other markers in combination with a measure of CRP may provide complementary information in this context. With respect to the practical application of such an algorithm, one approach would be to derive a score from the algorithm that provides the clinician with quartiles or tertiles of risk for PAD. Given that the current calculation of the panel score spans a narrow range, linear rescaling would be appropriate in order to provide a clinically relevant scale.

The association of fasting glucose with ABI was not surprising in view of the strong relationship between diabetes and PAD. However, many patients with or at risk of atherosclerosis have elevated fasting glucose prior to the diagnosis of diabetes, largely as a manifestation of insulin resistance. Thus, the use of fasting glucose is useful in this set-

ting and would be applicable to population-based screening. Lipid levels were assessed in our study, and they did not add to the predictive ability of the model (data not shown). This finding is consistent with other studies that have found that lipids are a better marker for CAD than PAD.

After adjustment for smoking, diabetes and age, the combination marker score was able to identify a group with an odds ratio of  $> 7$  for PAD in a population of patients referred for coronary angiography. Accordingly, this marker panel may be a useful diagnostic adjunct. Currently, clinical assessments of risk factor burden, such as the AHA risk score, incorporate 'traditional' CV risk factors and are used to predict risk of future events. To the extent that the AHA risk score reflects the burden of CV risk factors, it approximates the clinician's assessment of the risk of vascular disease. Accordingly, we compared the predictive power of the biomarker panel against the accepted AHA risk score. Individuals at highest risk were those with both a high AHA score, and a high biomarker panel score. Notably, there was a group of individuals with low AHA risk scores but high biomarker scores who were at high risk of PAD. Thus, the biomarker panel might identify a group of high-risk patients who would be missed using standard CV risk factor analysis.

The ABI is widely cited to have a specificity and sensitivity in the range of 95%. However, the sensitivity is reported to be lower (less than 80%) in a more recent study with angiographic confirmation.<sup>38</sup> These more recent data differ from earlier oft-quoted papers in that the earlier studies typically used patients with severe disease and compared them with young, healthy controls, thus eliminating borderline and mild disease and inflating the calculated sensitivity.

Nevertheless, the ABI provides a relatively simple and non-invasive test that has very good sensitivity and specificity when the test is performed by a trained individual. However, the time and space for the technician to perform the test are real costs. Furthermore, despite the availability of the ABI, and despite the urging of opinion-leaders for decades, the ABI has not been adopted by primary practitioners in their practices. Accordingly, PAD patients are underdiagnosed, and undertreated.<sup>9,39</sup> The primary practitioner lacks the specialized equipment and trained personnel to perform ABI measurements in the office setting. A blood test that increases the clinical index of suspicion could identify patients that merit greater scrutiny for PAD. Patients with elevated scores would be referred to vascular specialists who could provide further evaluation and appropriate management. In particular, intensive risk factor modification confers longevity in these patients and extends freedom from major adverse cardiovascular events.<sup>40</sup>

## Study limitations

Because the algorithm to evaluate the biomarker panel was trained on a single dataset, validation of this specific algorithm needs to be performed on an independent dataset. Furthermore, although this study confirms that cystatin C and  $\beta$ 2M are independent predictors of PAD,<sup>14</sup> the strength of the validation is diminished by the fact that the study population in the previous report is a subset of the current one. The c-statistic for the CAD + PAD versus CAD alone groups suggests that the biomarker panel may be a stronger discriminator of atherosclerosis generally rather than PAD specifically. Although this blood-based index may be helpful in identifying patients at increased risk for systemic atherosclerosis, further studies are needed to determine its association with morbidity and mortality in larger populations, including those from lower risk populations. If the panel proves useful in prognostication, it would be important to study the effects of medical therapy on the biomarker panel.

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## Conflict of interest statement

Drs Cooke, Wilson and Fung are inventors of patents based on findings from this study. The patents are jointly owned by Stanford University and Vermillion Inc. Vermillion Inc. provided supplies and equipment for this study.

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## Appendix Quartile cutoffs for individual markers and their combinations

Marker <sup>a</sup>	Quartile 1	Quartile 2	Quartile 3
β2M (mg/l)	1.49	1.87	2.60
Cystatin C (mg/l)	0.61	0.71	0.92
hsCRP (mg/l)	0.7	1.7	4.6
Glucose (mg/dl)	80.1	88.8	104.1
β2M + hsCRP	0.970851	0.984373	0.989266
β2M + cystatin C	0.965050	0.982961	0.995287
β2M + glucose	0.969806	0.990142	1.000673
hsCRP + cystatin C	0.973227	0.985754	0.992730
hsCRP + glucose	0.959438	0.993403	1.005873
Cystatin C + glucose	0.967480	0.992350	1.004795
β2M + hsCRP + cystatin C	0.961331	0.986804	1.003163
β2M + hsCRP + glucose	0.956735	0.994502	1.007900
hsCRP + cystatin C + glucose	0.955313	0.996475	1.010846
β2M + cystatin C + glucose	0.956829	0.992902	1.009818
β2M + hsCRP + cystatin C + glucose	0.949651	0.994995	1.015032

<sup>a</sup>Values for the combinations of markers are unitless panel scores calculated using equations derived from linear regression analysis.