Biomarkers of Peripheral Arterial Disease

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Abstract

Atherosclerotic arterial occlusive disease affecting the lower extremities is also known as peripheral arterial disease (PAD). This disorder affects 8 to 12 million individuals in the United States, and is also increasingly prevalent in Europe and Asia (1–4). Unfortunately, most patients are not diagnosed and are not optimally treated. A blood test for PAD, if sufficiently sensitive and specific, would be expected to improve recognition and treatment of these individuals. Even a biomarker panel of moderate sensitivity and specificity for PAD could refine risk-stratification, so as select individuals for diagnostic vascular examination. Alternatively biomarkers for PAD may be useful in determining prognosis or the risk for progression, or in determining the response to therapy. Finally, the discovery of biomarkers associated with PAD may provide novel insights into the pathophysiology of PAD, and new therapeutic avenues to pursue. Biomarkers may be derived from studies of the genome, transcriptome, proteome or metabolome. The focus of this review is on proteomic biomarkers associated with PAD.

Keywords
 Peripheral arterial disease; beta 2 microglobulin; C-reactive peptide; cystatin C; ankle-brachial index

Introduction

The prevalence of lower-extremity PAD, assessed using the ankle-brachial blood pressure index (ABI), has been estimated to be 10 to 20% of individuals over the age of 65 in community-based studies. Even greater prevalence is observed in individuals attending general medicine practices, where 20–30 percent of patients aged 50 and older have the disease (5,6). PAD causes limb pain with exertion, reduces functional capacity and quality of life (7) and is frequently associated with coronary, cerebral and renal artery disease (8). Individuals with PAD are at increased risk from acute cardiovascular events such as myocardial infarction, cerebrovascular attack, aortic aneurysm rupture, and vascular death, as well as ischemic ulceration and amputation (9,10). This increased risk for cardiovascular morbidity and mortality is seen even in patients without symptoms (11).

Aggressive medical treatment of risk factors can substantially reduce the mortality and morbidity of PAD (12). Unfortunately, PAD is under-diagnosed and under-treated, with most patients not receiving optimal management, which includes therapies proven to reduce mortality such as anti-platelet agents, statins and converting enzyme inhibitors (13). Suboptimal physician recognition and management of the condition is in part due to poor
public awareness of PAD; inadequate training and tools for primary physicians, and a lack of remuneration for screening; as well as the absence of the classic symptom complex in a majority of the patients. Classical intermittent claudication (i.e. exertional leg discomfort relieved by rest) is only noted by 10–30% of patients with PAD. Co-existing musculoskeletal disease or neuropathy commonly coexist with PAD and confound the clinical picture. Accordingly, clinical assessment for PAD has a relatively poor predictive value (<10%) (17). Structured questionnaires such as the Edinburgh Claudication Questionnaire have improved sensitivity and specificity when compared to clinician assessment (18) but these questionnaires only identify patients with classical symptomatology. Because the current recognition of PAD is suboptimal, and because effective therapy that improves mortality is available for these individuals, an efficacious strategy to screen the population for PAD is highly appealing.

**PAD: The Case for Screening**

By comparison to angiography, the ABI can detect hemodynamically significant lesions with a sensitivity in the range of 80–95%, and a specificity in the range of 95–100% (19,20). Furthermore, the ABI has independent prognostic value beyond the Framingham risk factors (21). The ABI is calculated from Doppler-derived measurements of the systolic pressure at the brachial and ankle arteries. By convention, for each lower extremity, the higher of the two ankle artery pressures is used for the ABI calculation. The ABI for that extremity is the higher ankle pressure divided by the higher of the two brachial artery pressures.

Targeted screening with ABI is recommended by all professional vascular societies, including the American College of Cardiology (22). The ACC/AHA guidelines support ABI screening in high-risk patients (defined as individuals <50 years of age with diabetes and one other atherosclerosis risk factor; individuals 50 to 69 years of age with a history of smoking or diabetes; individuals ≥70 years of age; those with leg symptoms with exertion or ischemic rest pain; and those with an abnormal lower-extremity pulse examination) (22). Also, the American Diabetes Association recommends annual screening for PAD in diabetics. (23).

Despite the abundant evidence supporting the value of the ABI; and despite careful studies that have revealed the suboptimal recognition of individuals with PAD and inadequate utilization of therapies that reduce mortality; there is resistance to adopting the ABI as a screening tool. The United States Preventive Services Task Force (USPSTF) has given the practice a “D” level recommendation (i.e. in their opinion, routinely providing the service to asymptomatic patients is ineffective, or harm from the test may outweigh benefits). Also, the American Academy of Family Physicians recommends against the use of the test in asymptomatic persons (24).

These opinions are contrary to the recommendations of vascular specialty societies, and have been convincingly rebutted (15). In brief, these unfortunate recommendations are driven by the concern that screening may lead to unnecessary tests, and increased risk from subsequent invasive studies or procedures. However, of much greater concern is the very real cost to the health care system and to the patient, of not identifying individuals with PAD. The primary purpose in screening for PAD is to identify individuals at high risk of vascular events (25) (9) (8) (26,27) so as to target them for aggressive risk reduction interventions (28) (29) (30) (31) (32) (33). Unfortunately, most PAD patients are currently not diagnosed, and not receiving therapies that can improve their prognosis (13) (34).
Beyond the ABI

Amongst vascular specialists there is widespread recognition of the value of the ABI, and evidence-based documentation of its sensitivity and specificity. However, a practical concern is that most primary practitioners lack the specialized equipment and trained personnel to perform ABI measurements in the office setting. In the absence of an effective screening strategy in the primary practitioner’s office, all individuals at risk could be referred for a formal vascular laboratory evaluation. This would be a costly screening strategy.

The number of individuals that should be screened for PAD (i.e. all smokers or diabetics over the age of 50, and all individuals over the age of 70), represents about 60 million individuals in the United States. An alternative screening approach would be to develop a blood biomarker, or panel of biomarkers, that could stratify the risk for individuals in the primary practitioners office. Such a panel could be assessed by a blood draw in the office, and would optimally identify a smaller subset of patients for vascular evaluation. Such an approach could reduce the overall cost of screening, while improving recognition and proper management. This alternative diagnostic paradigm requires progress toward developing novel biomarkers of PAD.

Challenges in discovering new biomarkers

There are hurdles to the discovery of any new blood protein biomarker. The most daunting problem is due to the great diversity of the proteome (i.e plasma contains approximately 10,000 plasma proteins and even more protein fragments), and its dynamic range (about ten orders of magnitude difference between the least and most abundant proteins) (35). The discovery process is complicated by the fact that the 22 most abundant proteins, such as albumin and the immunoglobulins, constitute approximately 99% of the total proteome mass (36). However, it is the low abundance proteins that are often of greatest interest as novel disease markers. Any technology to profile the plasma proteome in an informative manner must be able to delve deeply into the proteome, and to discriminate differences in the levels of low abundance proteins. For example, cardiac markers such as troponin are found in the nanomolar range while cytokines are in the femtomolar range.

Another important issue is that of confounding by medications or by associated diseases. Careful phenotyping of the subjects is critical for proteomic discovery, and the control group should be matched for variables already known to influence disease risk and outcome. Renal or hepatic disease may influence the excretion or metabolism or a biomarker. Other disorders may influence the level of a biomarker by pathophysiological processes unrelated to the disease of interest, eg. infection increases the plasma level of the cardiovascular biomarker C-reactive peptide. Technical details such as how the blood is drawn, processed and stored can substantially affect the findings, and lead to spurious results if the samples from different patient groups are not treated similarly. For example, multiple freeze-thaws while studying samples cause protein degradation, introducing artifactual peaks in mass spectroscopic analyses.

Despite these challenges, the field of cardiovascular proteomics continues to develop rapidly and a range of collaborative initiatives have been undertaken. The NIH/NHLBI has funded several centers for cardiovascular proteomics (37). The Human Proteome Organization has recently initiated a Plasma Proteome Project (38). The early phase of the PPP has reported the identification of approximately 345 cardiovascular disease-related proteins in human plasma (39). However, until recently there have been few attempts to detect biomarkers associated with PAD.
Do Biomarkers of PAD exist?

It could be argued that proteomic profiling is unlikely to yield plasma biomarkers specific for peripheral, as opposed to coronary artery disease (CAD). This is because PAD and CAD share common cardiovascular risk factors, and have many commonalities in pathophysiological processes. A number of biomarkers have been associated with PAD in population based studies (Table 1), such as inflammatory cytokines or chemokines, markers of endothelial dysfunction, mediators of angiogenesis or vascular regeneration, lipoproteins or lipid-associated proteins, indicators of oxidative stress or ischemia-reperfusion, metabolic modulators, and coagulation factors (40–45) (46).

However, none of these biomarkers are specific for PAD, being elevated in coronary artery disease (CAD) and other vascular disorders. This is not surprising, because these biomarkers were derived from “candidate protein”-based investigations that focused on proteins known to be associated with other vascular diseases or with pathophysiological mechanisms. Nevertheless, it is quite likely that pathological processes in the peripheral circulation will lead to the release of PAD-specific biomarkers. This is because there exist significant differences throughout the vasculature in endothelial and vascular smooth muscle gene expression (47) (48). Preclinical studies reveal functional differences as well; endothelium dependent vasodilation and smooth muscle vasoconstriction, in response to a variety of agonists, differ in coronary and limb arteries (49). Similarly in humans, endothelium-dependent responses in the peripheral arteries are quite different from those in central arteries (50). These differences in vascular reactivity between the peripheral and coronary arteries reflect differences in the expression of cell surface receptors and signaling pathways. There are also well-established differences between vascular beds in the expression of endothelial chemokines and adhesion molecules. These geographic differences are known to play a physiological role in lymphocyte homing and other cellular trafficking in immune surveillance (51). The phenotypic variation amongst vascular cells is a manifestation of their exposure to disparate milieus, their developmental divergence, and their persistent epigenetic differences (52,53).

We hypothesized that known and undiscovered phenotypic differences between peripheral and coronary vascular cells may affect their response to ischemia. Physiologists have long known that the stimulus of ischemia, followed by reperfusion, causes characteristic changes in a microcirculatory bed, including the release of adhesion molecules and production of inflammatory cytokines (54). We reasoned that chronic bouts of ischemia-reperfusion in the lower extremity of the claudicant might induce the expression and release of proteins characteristic of the peripheral circulation.

Approach to Discovery of Novel Biomarkers in PAD

Accordingly, we developed a discovery program for more specific biomarkers of PAD (55). Previous investigations of biomarkers for PAD have used a “candidate protein”-based approach. By contrast, to discover PAD-specific biomarkers, we employed an “agnostic” approach, using plasma proteomic profiling by mass spectroscopy (MS). We used a version of MS known as surface-enhanced laser desorption time-of-flight MS (SELDI-TOF MS). The advantage of SELDI-TOF MS is that it provides for a rapid chip-based segregation of peptides based on charge and lipophilicity. When combined with pH fractionation, the technique enhances MS resolution of the thousands of plasma proteins. Proteins in the sample are bound to the chip. An energy absorbing matrix is applied, re-dissolving the proteins on the chip, which co-crystallize with the matrix as it dries. Laser excitation ionizes the matrix causing it to release the proteins into the flight tube of a mass spectrometer, which subsequently strike a detector at the end of the tube. The resulting signal provides data from which the molecular weight and the amount of the peptide can be assessed. The
comparison of the spectra from cases and controls can lead to the identification of a peak that is different between the groups. This peak represents a protein that may be a new biomarker. Subsequent studies using complementary methods (MS-MS, immunoaffinity columns, ELISA, Western analysis) are done to confirm the identity of the protein.

We performed comprehensive proteomic profiling using this method in 88 patients with or without PAD. Our studies revealed several MS peaks, that were increased in PAD cases. Based upon its molecular weight, one of these peaks was thought to be beta 2 microglobulin. We chose this protein to study further as a proof of concept (55). In a confirmatory study using other proteomic approaches, ie. Western analysis and immunoaffinity studies, we found beta 2 microglobulin to be elevated in PAD subjects (and its plasma levels were inversely correlated with treadmill exercise capacity). Finally, we validated this biomarker using a nephelometric assay in a cross-sectional case-control study of patients undergoing elective coronary angiography for a diagnosis of coronary artery disease. We chose our cases and controls so that confounding clinical co-variates would contribute minimally to the difference between the two phenotypes. Cases had hemodynamically significant PAD as documented by reduced ankle-brachial indices (ABIs), whereas controls had normal ABIs. This study validated beta 2 microglobulin as a biomarker associated with PAD, independently of other cardiovascular risk factors.

**Biomarker Panels**

For any single biomarker (such as CRP), a certain percentage of subjects with abnormal levels will not have disease (false positive), whereas those with disease may have a normal levels (false negative). One approach to addressing this problem is to use a panel, in which each of the biomarkers contributes independent diagnostic information. Biomarker “panels” and index scores are beginning to be used in medicine to refine diagnosis and to aid in prognostication. For example, such index scores, incorporating novel biomarkers have been used to predict clinical outcomes in hepatocellular and breast malignancy (56) (57). Recently, Wang et al combined multiple biomarkers from the Framingham study to predict CVD outcomes and death (58).

In our discovery study, an additional biomarker candidate identified was cystatin C. A subsequent study of 540 high risk individuals revealed that β2M, cystatin C, hsCRP, and glucose were associated with PAD independently of the traditional risk factors of age, diabetes mellitus, hyperlipidemia, hypertension and tobacco use. Among the plasma markers tested, beta 2 microglobulin (beta2M) and cystatin C had the highest correlation with ABI, higher than any of the conventional risk factors of age, smoking status, and diabetes status. A biomarker panel score derived from beta2M, cystatin C, hsCRP, and glucose had an increased association with PAD status (59), independently of the traditional risk factors.

This biomarker panel is independent of, but only adds modestly to, the information provided by the Framingham risk factors. Although it is an imperfect prototype, this panel provides proof of concept for the promise of a multi-marker approach. Of note, a recent prospective cohort study of 1000 ambulatory elderly Japanese for eight years, revealed that beta2M, cystatin C and CRP were independent predictors of mortality (60). The most informative biomarker was beta2M. The area under the receiver operating characteristic curve for 8-year mortality was greatest for beta2M (0.70; 95% CI, 0.66–0.74), followed by cystatin C (0.66; 95% CI, 0.62–0.70) and CRP (0.57; 95% CI, 0.53–0.61). The authors concluded that serum beta2M is an independent predictor of total mortality in a general population of older adults and may be a better predictor than cystatin C or CRP.
Conclusion

There is a strong clinical need for more specific biomarkers for PAD. A blood test for PAD would increase recognition of the disease, and thereby improve clinical care. It is likely that a biomarker panel with high sensitivity and high specificity for PAD will be composed of biomarkers that circulate systemically, but which reflect the activity of local pathophysiological processes (Fig 3). To be clinically useful, the optimal biomarker(s) should be stable, easily measured, and should provide diagnostic or prognostic information that is incremental to existing biomarkers. The search for proteomic biomarkers is currently being driven by rapid advances in technology and bioinformatics that facilitate high throughput analysis of blood from clinical studies with sufficient sample sizes. The application of these technologies to PAD should be encouraged, as they are likely to yield useful diagnostics for PAD, as well as novel insights into the pathophysiology of the disease.

References


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Figure 1.
ROC analysis of conventional risk factors, the biomarker panel score, and a combination of conventional risk factors and the biomarker panel score.
Odds ratio of CAD+PAD status by AHA risk score and by biomarker panel score. There is a positive interaction between the two assessments of disease risk. Individuals were assigned an AHA risk score using the traditional cardiovascular risk factors as described (19). AHA risk scores of <5 (low), 5 to 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). n.s., not significant.

Figure 2.
Odds ratio of CAD+PAD status by AHA risk score and by biomarker panel score. There is a positive interaction between the two assessments of disease risk. Individuals were assigned an AHA risk score using the traditional cardiovascular risk factors as described (19). AHA risk scores of <5 (low), 5 to 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). n.s., not significant.
The optimal PAD blood test is likely to be comprised of a panel of biomarkers that circulate systemically, but which reflect the activity of local pathophysiological processes contributing to inflammation, oxidative stress, matrix remodeling, endothelial dysfunction, coagulation, metabolic perturbations and ischemia-reperfusion. Unique characteristics of the peripheral circulation may provide for specificity of the biomarkers that are released. In brackets are biomarkers that are elevated in PAD, but which are not specific. The identification of novel and specific biomarkers is underway. VEGF = vascular endothelial growth factor; Lp(a) = lipoprotein (a); B2M = beta 2 microglobulin; MMP9 = Matrix metalloproteinase 9; glu = glucose; sRAGE = soluble receptor for advanced glycosylation endproducts; sVCAM = soluble vascular cell adhesion molecule; TF = Tissue Factor; MCP-1 = Monocyte chemotactic protein 1; ADMA = asymmetric dimethylarginine; EPCs = endothelial progenitor cells; IL-6 = interleukin 6 (46,61,79–89)
### Table 1

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<thead>
<tr>
<th>Biomarkers Associated with PAD</th>
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<tr>
<td><strong>Inflammatory cytokines:</strong> CRP (42) ; Interleukin-6 (61,62)</td>
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<td><strong>Markers of endothelial dysfunction</strong> : ADMA (63–66); Soluble Cell Adhesion Molecules (CAMs) (67) (68) ; Von Willebrands Factor (69)</td>
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<td><strong>Modulators of Angiogenesis</strong> : Soluble Tie 2; VEGF; (46); Hepatocyte Growth Factor (70)</td>
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<td><strong>Lipoproteins</strong> : Oxidized LDL (40,41,71); Lipoprotein (a) (72)</td>
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<td><strong>Indicators of Oxidative Stress</strong> : Homocysteine (73) ; 8-Hydroxy-2-deoxy-2-deoxyguanosine (74); Isoprostanes (75)</td>
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<td><strong>Coagulation Factors</strong> : Thrombomodulin (76), D-Dimer, TPA, PAI-1, Fibrinogen (77,78)</td>
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<td><strong>Features of an “Ideal” PAD Biomarker</strong></td>
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<td>Sensitive for the Presence of Disease</td>
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<td>Specific, Good Negative Predictive Value</td>
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<td>Correlates with Prognosis</td>
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<td>Measurable with High Throughput Techniques</td>
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<td>Correlates with Disease Specific Features: ABI, Walking Time</td>
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<td>Levels Minimally or Predictably Affected by Confounding Factors</td>
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<td>Reproducible</td>
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