Fatty acid binding protein 3 is associated with peripheral arterial disease

Muzammil H. Syed, BSc, Abdelrahman Zamzam, BPharm, MSc, Hamzah Khan, BSc, Krishna Singh, PhD, Thomas L. Forbes, MD, FRCSC, Ori Rotstein, MD, MSc, Rawand Abdin, MD, John Eikelboom, MBBS, MSc, FRCPC, and Mohammad Qadura, MD, PhD, FRCSC Toronto, London, and Hamilton, Ontario, Canada

ABSTRACT

Background: Peripheral arterial disease (PAD) affects more than 150 million people worldwide and is associated with high rates of lower extremity amputation, myocardial infarction, stroke and death. Fatty acid binding protein 3 (FABP3) is released into circulation in patients with skeletal muscle injury. In this pilot study, we investigated a possible association between PAD and blood levels of FABP3.

Methods: Blood samples were collected from patients with clinical symptoms and diagnostic findings indicative of PAD (PAD group; ankle-brachial index [ABI] < 0.9; n = 75) and in those without clinical or diagnostic features of PAD (non-PAD group; ABI > 0.9; n = 75) presenting to vascular surgery ambulatory clinics at St. Michael’s Hospital. Plasma samples were analyzed by protein multiplex to quantify FABP3 levels.

Results: PAD patients were found to have higher blood levels of FABP3 compared to patients without PAD (mean 3.90 ± 1.69 vs 2.03 ± 0.78; P < .001). A subgroup analysis demonstrated that the FABP3 levels were increased by almost two-fold in patients with PAD, independent of coronary artery disease (P < .001) or diabetes mellitus status (P < .001). Moreover, a significant negative correlation between FABP3 and the ABI was observed in PAD and patients without PAD matched groups (r = −0.51; P = .001). Last, immunohistochemistry demonstrated elevated expressions of FABP3 within skeletal muscle obtained from patients with the most severe form of PAD, chronic limb-threatening ischemia, when compared with patients without PAD.

Conclusions: Patients with PAD have elevated plasma levels of FABP3. An increasing severity of PAD is associated with higher FABP3 levels. (JVS–Vascular Science 2020;1:168-75.)

Clinical Relevance: There is a pressing need for a simple, readily accessible, blood-based biomarker for PAD. In this study, we found elevated levels of FABP3 in patients with PAD. This increase in FABP3 was irrespective of history of coronary artery disease or diabetes. Furthermore, our data suggest that an increasing severity of PAD is associated with higher FABP3 levels. Subsequently, FABP3 may be a potential diagnostic biomarker for PAD. However, further studies are needed to confirm the capability of FABP3 to serve as a valid and reliable biomarker for PAD.

Keywords: Peripheral arterial disease; Biomarker; Fatty acid binding protein; Association

Lower extremity peripheral arterial disease (PAD) is the third most common presentation of atherosclerosis. More than 50% of patients with PAD are asymptomatic, and the remaining patients typically present with symptoms ranging from calf claudication to tissue loss. The wide spectrum of clinical presentations pose a challenge for PAD diagnosis, with community studies suggesting that physicians fail to diagnose PAD in about one-half of patients. This lack of awareness of the presence of PAD results in the failure of medical therapy initiation and atherosclerosis risk factor optimization, as well as deferred referral to specialists. It may also lead to a delay in surgical intervention, which puts patients at significant risk of morbidity, including major limb amputation, and mortality.
Physicians rely on clinical examination and diagnostic tools such as the ankle-brachial index (ABI) to diagnose patients with PAD. Although the ABI serves as a sensitive and cost-effective screening tool for PAD, primary health care providers report facing several barriers with using the ABI. Some of these barriers include time constraints, insufficient staff, limited equipment availability, lack of adequate training or availability of skilled personnel, and, in some cases, reimbursement. Furthermore, ABI values of patients may sometimes be falsely elevated owing to incompressible calcified vessels in diseases such as with diabetes with medial calcification of tibial vessels, systemic sclerosis, or rheumatic diseases.

Fatty acid binding protein 3 (FABP3) belongs to a family of multigene fatty acid binding proteins that is primarily expressed in skeletal and cardiac muscle. A small intracellular protein involved in fatty acid transfer and metabolism, FABP3 is released into the circulation when there is muscle injury. Furthermore, we previously tested the expression of several cardiac markers in patients with PAD and found a positive correlation between FABP3 and patients with symptomatic PAD. Therefore, we hypothesized that FABP3 levels are elevated in patients with PAD and might therefore be used as an aid to early diagnosis.

METHODS

Ethics approval. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and has been approved by the research ethics board at St. Michael’s Hospital—University of Toronto in Ontario, Canada. Informed consent (in written) was obtained from all participants.

Patient selection. For the purposes of this pilot study, the first encountered 75 patients with PAD and 75 patients without PAD presenting to ambulatory vascular surgery clinics at St. Michael’s Hospital between October 2017 and February 2018 were recruited. The diagnosis of PAD was established by an independent expert using clinical history, physical examination results, and lower limb arterial imaging as per the Rutherford Classification Criteria of chronic limb ischemia. The PAD cohort included patients of all severity levels ranging from claudication to chronic limb-threatening ischemia (CLTI). The non-PAD control cohort did not have a clinical history of claudication, had palpable distal pulses, and a normal arterial ultrasound examination of the lower limbs. We excluded patients (1) on anticoagulants, chemotherapy, or biological anti-inflammatory agents, (2) diagnosed with acute stroke or transient ischemic attack within 3 months, (3) with renal disease (stages 3, 4, and 5) according to the Kidney Disease Outcomes Quality Initiative clinical guidelines, and (4) with acute or a 3-month history of acute coronary syndrome as defined by American College of Cardiology or elevated troponins.

Baseline measurements. Recorded medical history included details of cardiovascular risk factors; prior coronary, cerebral, or peripheral artery disease; congestive heart failure; or cardiac arrhythmia as defined by American College of Cardiology. Hyperlipidemia was defined as total cholesterol of more than 5.2 mmol/L or triglyceride of greater than 1.7 mmol/L or the use of antihyperlipemic medication. Hypertension was defined as a systolic blood pressure of 130 mm Hg or greater or a diastolic pressure of 80 mm Hg or greater, or the use of an antihypertensive medication. Diabetes mellitus was defined as a glycosylated hemoglobin A1c of 6.5% or greater or the use of antidiabetic medication. Smoking status was recorded for each patient.

Each patient underwent lower limb arterial imaging with ultrasound examination, ABI, or toe-brachial index (TBI). Blood samples were drawn into vacutainer tubes containing EDTA. After centrifugation at 3000 rpm for 10 minutes (4°C), plasma was aliquoted and stored at −80°C.

FABP3 multiplex assay. Plasma samples were analyzed in duplicate using MILLIPLEX MAP Human Cardiovascular Disease (CVD) Magnetic Bead Panel 1 (EMD-Millipore; Billerica, Mass) to determine the concentrations of FABP3. All sample analyses were completed on the same day to eliminate inter-assay variability. Sample intra-assay and inter-assay coefficient of variation were both less than 10%. The MagPix analyzer (Luminex Corp; Austin, Tex) was calibrated prior to analysis using Fluidics Verification and Calibration bead kits (Luminex Corp). A minimum of 50 beads for each targeted biomarker were acquired using Luminex xPonent software and analyzed using Milliplex Analyst software (v.5.1; EMD-Millipore).

Sample processing. Muscle biopsies collected from patients were processed immediately. Each specimen was divided into two pieces. The first muscle piece was fixed in formalin and further processed into
paraffin-embedded blocks for immunohistochemistry purposes, whereas the second muscle piece was frozen immediately in liquid nitrogen for Western blot studies.

**Immunohistochemistry of FABP3 biomarkers.** Three sections of 5 μm thickness were cut from each paraffin block. Medial head gastrocnemius muscle biopsies were obtained from three patients with CLTI undergoing major limb amputation (experimental group) and three patients without PAD undergoing elective surgery to manage varicose veins (negative control). As a positive control, left atrial appendages were obtained from three patients undergoing open heart surgery for acute coronary syndrome. Thereafter, all slides were evaluated with standard protocols for staining with hematoxylin and eosin (Sigma, St. Louis, Mo). For detection of FABP3, sections were stained using antihuman mouse polyclonal FABP3 antibody (Thermo Fisher Scientific, Waltham, Mass) and then incubated again with horseradish peroxidase-conjugated secondary antibody according to the standard immunostaining procedure.

**Western blot.** All snap frozen muscle samples were homogenized in a lysis buffer (Cell Signaling Technology, Beverly, Mass) using a ultrasonic homogenizer (Biologics Inc, Manassas, Va). Protein concentration was measured in duplicate by the bicinchoninic acid assay (Pierce, Rockford, Ill). In these studies, aliquots equivalent of approximately 40 μg of protein were separated on gradient (10%-15%) polyacrylamide sodium dodecyl sulphate gels. After electrophoresis, proteins were electrotransferred to a nitrocellulose membrane. The membrane was blocked with skimmed milk powder in TBST (0.05% Tween 20, 100 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.8) for 30 minutes and then incubated with the primary antibody FABP3 (Abcam, Toronto, ON, Canada) overnight at 4°C. Secondary antibody polyclonal goat antimouse horseradish peroxidase conjugated (Cedarlane, Burlington, ON, Canada) was added. Blots were developed in ECL detection reagents (Amersham [Little Chalfont. UK], ECL Western Blotting Detection Reagents; GE Healthcare [Boston, Mass]) and the chemiluminescence emitted from immune complexes was visualized with a ChemiDoc image system (Bio-Rad, Mississauga, ON, Canada). Images were quantified using Image J software.

**Statistical methods.** Continuous variables were summarized using means and standard deviation. Categorical variables were summarized as number and proportion. Baseline characteristics were compared between the groups with and without PAD using independent t-tests or the Mann-Whitney U test for continuous variables. Fisher’s exact test or χ² test was used for categorical variables. The Kruskal-Wallis test was used to determine whether there were any significant differences between more than two independent groups. Furthermore, using propensity score adjustment method, patients with and without PAD were matched based on age, coronary artery disease, stroke, smoking history, and diabetes. Using the matched groups, correlation analysis between FABP3 and ABI was performed. SPSS software, version 23 (SPSS Inc., Chicago, Ill) was used for data entry and analysis. All analyses were carried out at a 5% two-sided significance level.

**RESULTS**

**Cohort description.** The PAD cohort (n = 75) was mainly composed of males who had a higher prevalence of cardiovascular risk factors compared with the non-PAD patients (n= 75; Table I). Thus, patients with PAD were older; more often had hypertension, hypercholesterolemia, diabetes, smoking, and a history of coronary arterial disease (CAD), and had lower ABI values.

**FABP3 levels increase in patients with PAD.** Patients with PAD had significantly higher blood levels of FABP3 compared with patients without PAD (mean 3.90 ± 1.67 vs mean 2.27 ± 0.32, respectively: P < .0001). Furthermore, since FABP3 has been previously suspected to be associated with diabetes mellitus and CAD, we investigated the association between FABP3 levels in PAD and patients without PAD with and without these cardiovascular risk factors.22 As shown in Fig 1, A, patients with PAD had higher levels of FABP3 than those without PAD irrespective of whether they had a history of CAD (mean, 4.15 ng/mL ± 1.83 vs mean, 2.40 ng/mL ± 0.61) or no history of CAD (mean, 4.08 ng/mL ± 1.77 vs mean, 2.11 ng/mL ± 0.72) (P for interaction = .832). A similar pattern was evident for PAD compared with patients without PAD who had diabetes (mean, 4.22 ng/mL ± 1.12 vs mean, 2.28 ng/mL ± 0.66) and in those with no diabetes (mean, 4.00 ng/mL ± 0.98 vs mean, 2.15 ng/mL ± 0.72) (P for interaction = .644; Fig 1, B).

**FABP3 levels in increased severity of PAD disease and its association with ABI.** The relationship between worsening PAD severity and FABP3 was also investigated. PAD severity was stratified based on the ABI values into (1) control patients without PAD (1.3 ≤ ABI ≤ 0.9), (2) mild to moderate PAD (0.9 < ABI < 0.4), (3) severe PAD (ABI ≤ 0.40), and (4) patients with noncompressible (NC) tibial vessels (ABI > 1.3). In the NC group, the PAD severity was based on the TBI (n = 5). A TBI value of less than 0.67 was considered abnormal. All of patients in the NC group had a TBI of less than 0.67 with a mean TBI of 0.3. When compared with control patients without PAD, FABP3 levels were higher with increasing severity of PAD (Fig 2, A). FABP3 levels were highest in the NC group, followed by severe PAD, mild to moderate PAD, and control patients without PAD (P for trend < .001).

We also performed a correlation study between FABP3 levels and ABI (which reflects PAD status), among matched patients with PAD (n = 35; ABI <0.9) and
patients without PAD (n = 35; ABI ≥ 0.9; Table II). As expected, there were no significant differences between non-PAD and PAD groups in the measured risk factors (age, sex, coronary artery disease, hypercholesteremia, hypertension, smoking history, and diabetes) except for ABI. Relative to patients without PAD, FABP3 levels
were significantly higher in patients with PAD (3.9 ± 1.6 ng/mL; non-PAD, 2.2 ± 0.6 ng/mL; \( P < .001 \)). After applying spearman’s correlation test on this matched cohort, the plasma FABP3 levels were significantly inversely correlated with ABI (\( r = -0.51; \ P = .001 \); Fig 2, B).

Localization of FABP3 within lower limb skeletal muscles. Muscle biopsies were obtained from three patients with CLTI undergoing major limb amputation, three patients without PAD undergoing elective lower limb surgery to manage varicose veins (negative control), and three patients undergoing open heart surgery for acute coronary syndrome (positive control). Our results demonstrated higher levels of FABP3 in patients with CLTI as compared with control patients without PAD (Fig 3). Using Western blot studies, FABP3 expression was investigated in gastrocnemius muscle obtained from the control group without PAD and the experimental CLTI patient group. A quantitative analysis revealed a significant increase in the expression of FABP3 in muscle tissue obtained from patients with CLTI, when compared with control patients without PAD (Fig 4). This finding is very interesting, because this experiment demonstrates an increase in FABP3 protein expression within muscle biopsies obtained from patients with CLTI.

**DISCUSSION**

In this pilot study, we found that patients with PAD had significantly higher blood levels of FABP3 than those without PAD. This association was evident irrespective of whether patients had a history of CAD or diabetes. Furthermore, FABP3 levels correlated with disease severity, with higher levels in those with more advanced PAD. We also found that muscle biopsies obtained from patients with CLTI compared with control patients without PAD had higher FABP3 protein expression.

FABP3, also known as heart-type FABP, belongs to a family of multi-gene fatty acid binding proteins.\(^{23}\) FABP3 is primarily expressed in skeletal and cardiac muscle, where it constitutes 4% to 5% of all cellular proteins, but it is also found in the brain.\(^{15,16}\) Because of the abundance of FABP3 in the myocardium and its rapid release into the circulation after the onset of cardiomyocyte damage, FABP3 has been used as a biomarker of acute coronary artery syndrome.\(^{14}\) Recently, FABP3 has also been shown to be potentially linked to the progression of atherosclerosis owing to its contributing role in endothelial dysfunction.\(^{24}\) Within muscle cells, FABP3 is found mainly within the cytosol,\(^{25}\) where it mediates the uptake of intracellular fatty acids as well as their transport to the mitochondrial \( \beta \)-oxidation system.\(^{26}\) During physical exercise, there is an increased demand of fatty acid use,\(^{27}\) and this demand results in increased FABP3 expression.\(^{28}\) Prior studies have reported the release of FABP3 from skeletal muscle in patients with myopathy, after exercise or skeletal muscle injury.\(^{17–20}\)

Patients with PAD are considered to have an acquired form of myopathy caused by repetitive tissue ischemia and reperfusion\(^{19–52}\) that is characterized by alterations in the expression of mitochondrial enzymes, deregulation of mitochondrial respiration, increased mitochondrial oxidative stress, and muscle apoptosis.\(^{33–35}\) This
Table II. Patient demographics and clinical characteristics for the matched groups with and without peripheral arterial disease (PAD)

|                         | No PAD (n = 21) | PAD (n = 21) | P value |
|-------------------------|-----------------|--------------|---------|
| ABIa                    | 11 ± 0.1        | 0.6 ± 0.2    | <.001   |
| Agea                    | 69 ± 8.3        | 70 ± 10.2    | .95     |
| Sex (male)b             | 17 (81)         | 12 (57)      | .09     |
| Hypertensionb           | 13 (62)         | 16 (76)      | .32     |
| Hypercholesterolemia b  | 17 (81)         | 11 (52)      | .05     |
| Diabetesb               | 7 (33)          | 10 (48)      | .35     |
| Smoking historyb        | 16 (76)         | 14 (67)      | .50     |
| Coronary artery diseaseb| 13 (62)         | 13 (52)      | .53     |
| Stroke/TIAb             | 1 (5)           | 4 (19)       | .15     |

ABI, Ankle-brachial index; TIA, transient ischemic attack.
Values are mean ± standard deviations (calculated for continuous variables) or number (%; calculated for categorical variables); all numbers were rounded to one decimal place.

aDifferences between groups were compared using the Mann-Whitney U test.

bDifferences between groups were compared using χ² test.

**Fig 3.** Fatty acid binding protein 3 (FABP3) is expressed in the skeletal muscles of patients with peripheral arterial disease (PAD). Hematoxylin and eosin staining (A, C, and E) and FABP3 staining (B, D, and F) were used to assess cellular histology and localization of FABP3 in cardiac myocytes obtained from patients with acute coronary syndrome (B) as well as skeletal muscles obtained from patients without PAD (D) and patients with chronic limb-threatening ischemia (CLTI) (F).
process can potentially result in the release of FABP3 into circulation; however, additional studies are needed to confirm this finding. Our findings of elevated FABP3 blood levels in patients with PAD, and of a correlation between FABP3 levels and severity of PAD disease, are consistent with previously published studies demonstrating FABP3 release from patients with myopathy.36,37 Thus, our data suggest that FABP3 is released into plasma at increased levels with worsening PAD disease status. However, further studies are needed to confirm this hypothesis.

Our pilot study has some limitations. First, we included only a modest number of patients, which limited the power to adjust for confounders. Second, we measured FABP3 at only one time point and thus we are unaware of whether blood levels change over time. Third, we excluded patients with acute coronary ischemia and those with moderate to severe kidney disease. Fourth, we are uncertain whether FABP3 is associated with PAD after treatment or independent of treatment.

In conclusion, we have demonstrated an association between circulating blood levels of FABP3 and the presence and severity of PAD. Although the findings are robust and the association seems to be independent of other factors that may elevate blood levels of FABP3 within this patient population, they require replication in larger cohorts that include a broader range of patients with PAD and with other comorbidities. If our findings are confirmed, FABP3 may be a valuable biomarker to aid primary care physicians in screening patients for PAD, evaluating disease severity, and in making decisions regarding the initiation of medical therapy, thereby helping to further reduce the burden of morbidity and mortality in this undertreated population.

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**AUTHOR CONTRIBUTIONS**
Conception and design: RA, MQ
Analysis and interpretation: MS, AZ, KS, TF, OR, RA, JE, MQ
Data collection: HK, MQ
Writing the article: MS, RA, MQ
Critical revision of the article: AZ, HK, KS, TF, OR, RA, JE, MQ
Final approval of the article: MS, AZ, HK, KS, TF, OR, RA, JE, MQ
Statistical analysis: AZ, RA, MQ
Obtained funding: MQ
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