diabetology

MDPI

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Identification of Inflammatory Biomarkers for Predicting Peripheral Arterial Disease Prognosis in Patients with Diabetes

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Abstract: Background: Peripheral arterial disease (PAD) is known to be strongly linked to major adverse limb events, ultimately leading to an increased risk of limb-threatening conditions. We developed a predictive model using five identified biomarkers to predict major adverse limb events, limb loss, diabetic (DM) foot ulcers, and vascular intervention in patients with underlying PAD and DM over 2 years. Methods: A single-center prospective case control study with was conducted with 2 years' follow up. In the discovery phase the cohort was randomly split into a 70:30 ratio, and proteins with a higher mean level of expression in the DM PAD group compared to the DM non-PAD group were identified. Next, a random forest model was trained using (1) clinical characteristics, (2) a five-protein panel, and (3) clinical characteristics combined with the five-protein panel. Demographic data were analyzed by independent *t*-test and chi-square test. The importance of predictive features was calculated using the variable importance (gain) score. The model was used and assessed for its ability to diagnose PAD, predict limb loss, predict major adverse limb events (MALEs), predict diabetic foot ulcers, and predict the need for vascular surgery. The model was evaluated using area under the receiver operating characteristic curve and net reclassification index. Results: The cohort of 392 patients was matched for age, sex, and comorbidities. Five proteins were identified (TNFa: tumor necrosis factor alpha, BMP-10: bone morphogenic protein 10, CCL15/MIP1 delta: chemokine (c-c motif) ligand 15/macrophage inflammatory protein 1 delta, MMP-10: matrix metalloprotease 10, and HTRA2/Omi: HTRA2, also known as Omi) as having a significantly higher level of expression in the DM PAD group. HTRA/Omi had the highest contribution to the model's ability to diagnose PAD in diabetic patients. Model performance was best when combined with clinical characteristics to predict limb loss (AUROC 0.86, 0.76, 0.80), foot ulcer (AUROC 0.87, 0.82, 0.67), MALE (AUROC 0.81, 0.78, 0.67), and the need for vascular surgery (AUROC 0.82, 0.81, 0.61). Conclusions: In this study, we describe a biomarker panel that can be used in combination with clinical characteristics to create an accurate prediction model for diagnosis and prognostication of PAD in the setting of DM.

Academic Editor: Federico Biscetti

Received: 27 November 2024
Revised: 24 December 2024
Accepted: 25 December 2024
Published: 30 December 2024

Citation: Draper, K.; Li, B.; Syed, M.; Shaikh, F.; Zamzam, A.; Abuhalimeh, B.J.; Rasheed, K.; Younes, H.K.; Abdin, R.; Qadura, M. Identification of Inflammatory Biomarkers for Predicting Peripheral Arterial Disease Prognosis in Patients with Diabetes. *Diabetology* **2025**, *6*, 2. https://doi.org/10.3390/diabetology6010002

Keywords: peripheral arterial disease; diabetes; biomarkers

1. Introduction

Peripheral arterial disease (PAD) is an underdiagnosed condition resulting from atherosclerosis. Many patients may be asymptomatic, whereas others may present with mild symptoms that could be easily mistaken for the normal aging process. Some patients present with claudication, non-healing ulcers, or necrotic skin lesions [1]. Diabetes mellitus (DM) is a major risk factor for PAD [2–4], and the prevalence of DM is rising, with a projected increase to 693 million people by the year 2045 [5].

The combination of DM and PAD has a synergistic effect, resulting in higher rates of morbidity, mortality, and amputation compared to patients with PAD or DM alone [6–8]. Ankle brachial index (ABI) remains the main modality to screen for PAD in patients with DM [9]. Due to incompressible arteries, neuropathy, and diabetic foot ulcers, ABI can be unreliable or unmeasurable in patients with DM [9]. Therefore, it is important to identify alternative ways to screen for PAD in patients with diabetes.

Protein biomarkers have been identified in patients with PAD and DM such as adiponectins, multiple growth factors, interleukins, metalloproteinases, and others [10–16]. A specific and consistent group of biomarkers seen across multiple studies for the combination of PAD and DM has not emerged, but common themes of inflammatory states, hypercoagulability, and vascular endothelial dysfunction are associated with the currently identified proteins [14–16].

In this study we identified five proteins that were expressed more in patients with PAD and DM compared to patients with DM alone. We then developed a panel of these five proteins and evaluated the panel's ability to diagnose PAD in patients with DM as well as predict major adverse limb events, limb loss, diabetic foot ulcers, and the need for vascular intervention in patients with DM and PAD over a 2-year follow-up period.

2. Methods

2.1. Ethics Approval

This study was granted approval by the research ethics board at Unity Health Toronto, University of Toronto, Canada. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Unity Health Toronto, University of Toronto, Canada, on 8 February 2017 (REB # 16-365). All patients provided informed consent to participate in this study.

2.2. Study Design and Patients

This was a prospective case control study where patients with diabetes presenting to St. Michael's Hospital vascular surgery clinics between March 2019 and February 2022 were recruited. Diagnostic criteria for diabetes were based on the Diabetes Canada guidelines [17], including HbA1C $\geq$ 6.5, fasting plasma glucose $\geq$ 7 mmol/L, 2 h plasma glucose 75 g oral glucose tolerance test $\geq$ 11.1 mmol/L or random plasma glucose $\geq$ 11.1 mmol/L, or taking antidiabetic medication. Patients were then divided into two groups based on whether or not they had PAD. PAD was defined as ankle brachial index (ABI) < 0.9, toe brachial index (TBI) < 0.67, or absent/diminished pulses [18]. Patients were excluded if they had acute limb ischemia, acute coronary syndrome, or elevated troponin or had taken biological anti-inflammatory medications within the previous 3 months.

2.3. Baseline Demographic and Clinical Characteristics

Baseline variables were collected, including age, gender, smoking status (non-smoker, current smoker, past smoker), history of hypertension (systolic blood pressure $\geq$ 130 mmHg, diastolic blood pressure $\geq$ 80 mmHg, or taking blood pressure-lowering therapy), hypercholesterolemia (total cholesterol > 5.2 mmol/L, triglyceride > 1.7 mmol/L, or taking lipid-lowering therapy), congestive heart failure, coronary artery disease, stroke, and medications (statin, angiotensin-converting enzyme inhibitor [ACE-i]/angiotensin receptor blockers [ARB], beta blocker [B-bl], calcium channel blocker [CCB], diuretic, insulin, oral hypoglycemic, and acetylsalicylic acid [ASA]). Definitions for cardiovascular risk factors were based on American College of Cardiology guidelines [19].

2.4. Protein Collection and Identification

Blood samples were collected from study participants and measured using a commercially available LUMINEX assay (Bio-Techne, Minneapolis, MN, USA) according to the manufacturer's instructions [20]. The following proteins were identified to play a role in peripheral arterial disease, atherosclerosis, and cardiovascular disease: tumor necrosis factor alpha (TNFa), bone morphogenic protein 10 (BMP-10), chemokine (c-c motif) ligand 15/macrophage inflammatory protein 1 delta (CCL15/MIP1 delta), matrix metalloprotease 10 (MMP-10), and HtrA serine peptidase 2, also known as Omi (HTRA2/Omi). Prior to sample analysis, Fluidics Verification and Calibration bead kits (Luminex Corp, Austin, TX, USA) [21] were used to calibrate the MagPix analyzer (Luminex Corp; Austin, TX, USA) [22]. To prevent any inter-assay variability, all sample analyses were carried out on the same day. Sample intra-assay and inter-assay coefficients of variability were <10%. At least 50 beads for each protein were acquired and analyzed using Luminex xPonent software, version 4.3 [23].

2.5. Model Development and Evaluation

The study cohort was randomly split into a 70:30 ratio, and proteins with a higher mean level of expression in the DM and PAD group compared to the DM non-PAD group were identified. These proteins included TNFa, BMP-10, CCL15/MIP-1 delta, HTRA2/Omi, and MMP-10. The five-protein panel was further investigated as a potential panel for diagnosing PAD in diabetic patients.

Next, a random forest model was trained using input features of clinical characteristics (age, gender, history of hypertension, dyslipidemia, diabetes, past/current smoking, congestive heart failure, coronary artery disease, previous stroke, claudication, ABI, ASA, statins, ACE-i/ARB, B-Bl, CCB, hydrochlorothiazide (HCTZ) or furosemide, oral antihyperglycemic agent, and insulin), the five-protein panel, and clinical characteristics combined with the five-protein panel. The model was assessed using net reclassification improvement (NRI) to quantify the model's ability to reclassify subjects [24]. After training, the models were further assessed by calculating the area under the receiver operating characteristic curve (AUROC). The importance of predictive features was calculated using the variable importance (gain) score, which indicates the predictive impact of a specific covariate [25].

Using random forest machine learning, performance metrics for diagnosing PAD, as well as for predicting major adverse limb events (MALEs), limb loss, tissue necrosis, development of diabetic foot wounds, and the need for vascular intervention were assessed based on different inputs. The model inputs that were assessed included clinical characteristics alone, the five-protein panel alone, and clinical characteristics combined with the five-protein model.

2.6. Follow-Up and Outcomes

Patients were followed up with at 1 year and 2 years. During follow-up they were assessed with a complete history, physical exam, ABIs, and study outcomes. The primary outcome of this study was MALE, defined as need for vascular intervention or major amputation within a 2-year period. The secondary outcome was development of a diabetic foot ulcer within the 2-year follow-up period.

2.7. Statistical Analysis

Clinical and demographic data are reported as means and standard deviations (SDs) or numbers. The independent *t*-test was used to analyze continuous variables, and the chi-square test was used for categorical variables. The difference in protein levels in the PAD and DM group vs. the PAD and non-DM group was calculated by independent *t*-test (if normally distributed) or Mann-Whitney U test (if non-normally distributed). Clinical events at 2 years were compared between the PAD with diabetes patients and the PAD without diabetes patients using the chi-square test. Model performance was assessed by calculating the AUROC and Net Reclassification Index. Absence of MALE over a 2-year period was evaluated with Kaplan–Meier curves. A two-tailed p value < 0.05 was considered significant. SPSS software version 23 was used to conduct the statistical analyses (SPSS Inc., Chicago, IL, USA) [26].

3. Results

3.1. Patient Characteristics

A total of 392 diabetic patients were recruited for this study. This group comprised patients with diabetes and no peripheral arterial disease (DM non-PAD) (n = 101) and patients with diabetes and peripheral arterial disease (DM PAD) (n = 291). The DM PAD patients were older than the DM non-PAD patients (69 ± 8 years vs. 66 ± 13 years, $p = 0.001$). They were also more likely to be on a statin (70% vs. 57%, $p < 0.001$), ACE-i/ARB (69% vs. 45%, $p = 0.001$), beta blocker (46% vs. 32%, $p = 0.001$), and aspirin (83% vs. 59%, $p < 0.001$) (Table 1). There was no difference in the remainder of the baseline characteristics, including sex, hypertension, hypercholesterolemia, smoking status, presence of coronary artery disease, history of stroke, or use of diabetic medications (Table 1).

Table 1. Baseline characteristics of patients with diabetes and no peripheral artery disease compared to patients with diabetes and peripheral arterial disease.

Characteristic	DM and Non-PAD (n = 101)	DM and PAD (n = 291)	*p*
Age, mean (SD)	66 (8)	69 (13)	0.001
Sex, male	80 (75)	214 (75)	0.948
Hypertension	87 (81)	250 (88)	0.104
Hypercholesteremia	92 (86)	247 (87)	0.860
Past smoking	50 (47)	147 (52)	0.217
Current smoking	21 (20)	67 (24)	0.217
Congestive heart failure	5 (5)	20 (7)	0.397
Coronary artery disease	36 (34)	132 (46)	0.024
Stroke	18 (17)	49 (17)	0.931
Statin	58 (57)	203 (70)	<0.001

Table 1. *Cont.*

Characteristic	DM and Non-PAD (n = 101)	DM and PAD (n = 291)	*p*
ACEi/ARB	45 (45)	200 (69)	0.001
B-bl	32 (32)	134 (46)	0.001
CCB	22 (22)	78 (27)	0.076
HCTZ, lasix	11 (11)	38 (13)	0.286
Insulin	40 (4)	17 (6)	0.404
Oral hypoglycemic	6 (6)	20 (7)	0.830
ASA	59 (59)	241 (83)	<0.001

Values reported as N (%) unless otherwise indicated. SD: standard deviation, ACE-i: angiotensin-converting enzyme inhibitor, ARB: angiotensin receptor blocker, B-bl: beta blocker, CCB: calcium channel blocker, HCTZ: hydrochlorothoazide, ASA: asytylcylic acid.

3.2. Protein Expression and Identification

After randomly splitting the study cohort into a 70:30 ratio, five proteins were found to have higher mean levels of expression (±SD) in the DM PAD group compared to the DM non-PAD, including TNFa (DM non-PAD = 3.04, DM PAD 4.19, $p = 0.001$), BMP-10 (DM non-PAD = 0.84, DM PAD 1.35, $p = 0.029$), CCL15/MIP-1 delta (DM non-PAD = 1.67, DM PAD 2.54, $p = 0.029$), HTRA2/Omi (DM non-PAD = 0.57, DM PAD 0.88, $p = 0.016$), and MMP-10 (DM non-PAD = 0.46, DM PAD 0.64, $p = 0.032$), as summarized in Table 2.

Table 2. Protein expression levels were significantly higher in the diabetic PAD group.

Group	Protein	Non-Pad	PAD	*p*
Non-diabetic	TNFa	4.20	4.51	0.301
	BMP-10	0.13	0.13	0.993
	CCL15/MIP-1 delta	1.47	1.52	0.760
	HTRA2/Omi	1.04	1.15	0.863
	MMP-10	0.58	0.76	0.004
Diabetic	TNFa	3.04	4.19	0.001
	BMP-10	0.84	1.35	0.029
	CCL15/MIP-1 delta	1.67	2.54	0.001
	HTRA2/Omi	0.57	0.88	0.016
	MMP-10	0.46	0.64	0.032

TNFa: tumor necrosis factor alpha, BMP-10: bone morphogenic protein 10, CCL15/MIP1 delta: chemokine (c-c motif) ligand 15/macrophage inflammatory protein 1 delta, HTRA2/Omi: HTRA2, also known as Omi, MMP-10: matrix metalloproteinase 10.

3.3. Model Performance for Diagnosing PAD

Based on findings from the discovery phase, we further investigated the ability of the five-protein panel to diagnose PAD in diabetic patients. The random forest model exhibited the following performance metrics for diagnosing PAD using different input features: clinical characteristics alone (AUROC 0.84), five-protein panel alone (AUROC 0.87), and clinical characteristics combined with the five-protein panel (AUROC 0.88). The addition of the protein panel significantly enhanced the model's performance compared to using clinical characteristics alone, resulting in a Net Reclassification Index (NRI) of 0.28. This improvement was statistically significant, with a *p*-value of 0.027 (Figure 1).

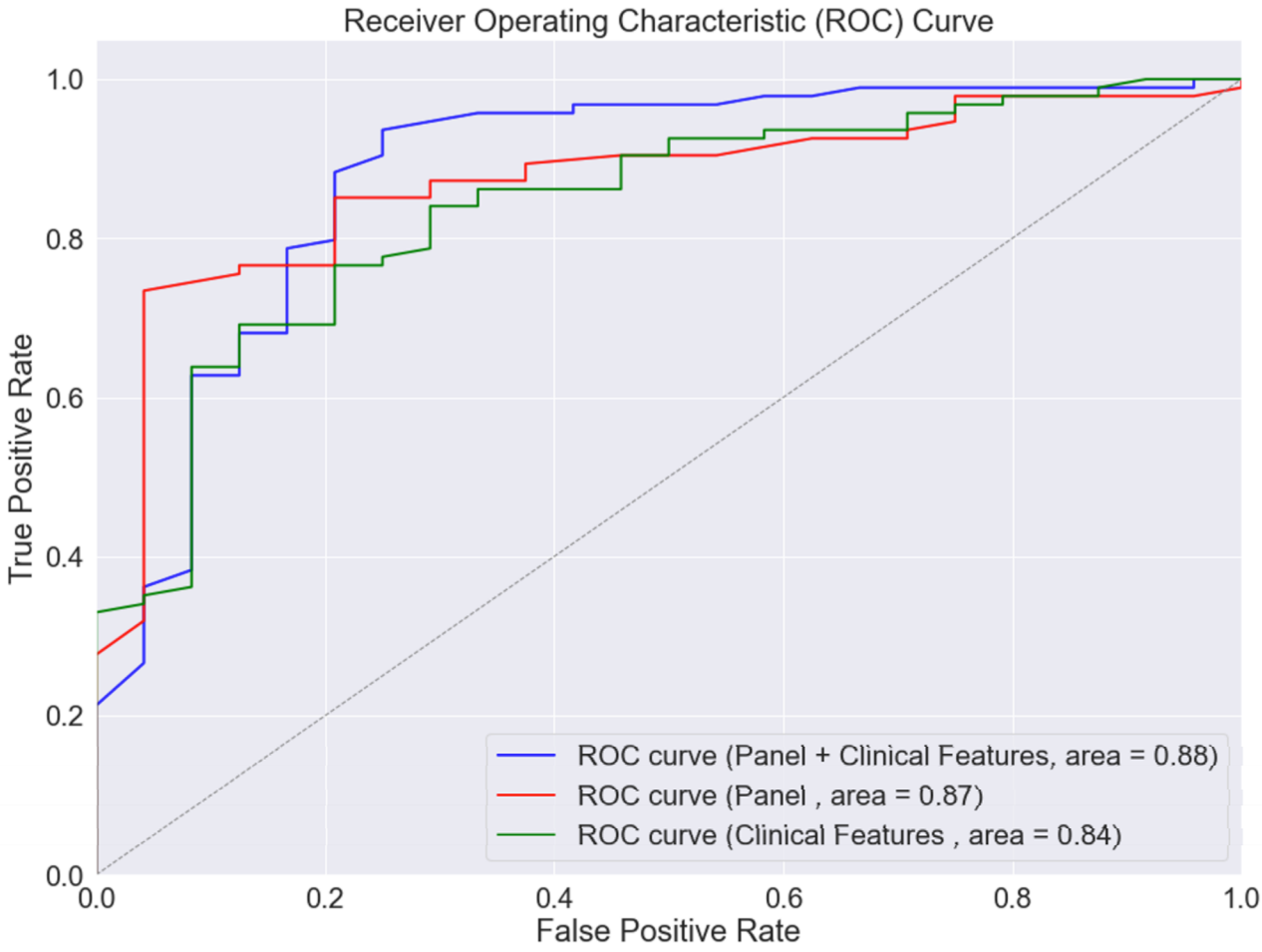

Figure 1. Random forest model performance metrics for diagnosing peripheral artery disease in patients with diabetes. The inputs included: clinical characteristics alone (AUROC 0.84), the five-protein panel alone (AUROC 0.87), and clinical characteristics combined with the five-protein panel (AUROC 0.88). ROC: receiving operator characteristic, AUROC: area under the receiver operating characteristic.

3.4. Model Performance for Predicting Limb Loss at 2 Years

The random forest model's performance for predicting limb loss at 2 years was evaluated using different input features: clinical characteristics alone (AUROC 0.80), the five-protein panel alone (AUROC 0.76), and clinical characteristics combined with the five-protein panel (AUROC 0.86). Incorporating the protein panel enhanced the model's predictive performance, resulting in an NRI of 0.23, and this was statistically significant (p = 0.002) (Figure 2). The most significant protein for predicting limb loss at 2 years was TNF alpha (Figure 3).

3.5. Model Performance for Predicting Need for Vascular Surgery at 2 Years

The random forest model's performance for predicting the need for vascular surgery (revascularization) at 2 years was evaluated using different input features: clinical characteristics alone (AUROC 0.67), the five-protein panel alone (AUROC 0.82), and clinical characteristics combined with the five-protein panel (AUROC 0.87). Incorporating the protein panel enhanced the model's predictive performance, resulting in an NRI of 0.39, and this was statistically significant (p = 0.015) (Figure 4). The most significant protein for predicting need for vascular surgery at 2 years was TNFa (Figure 5).

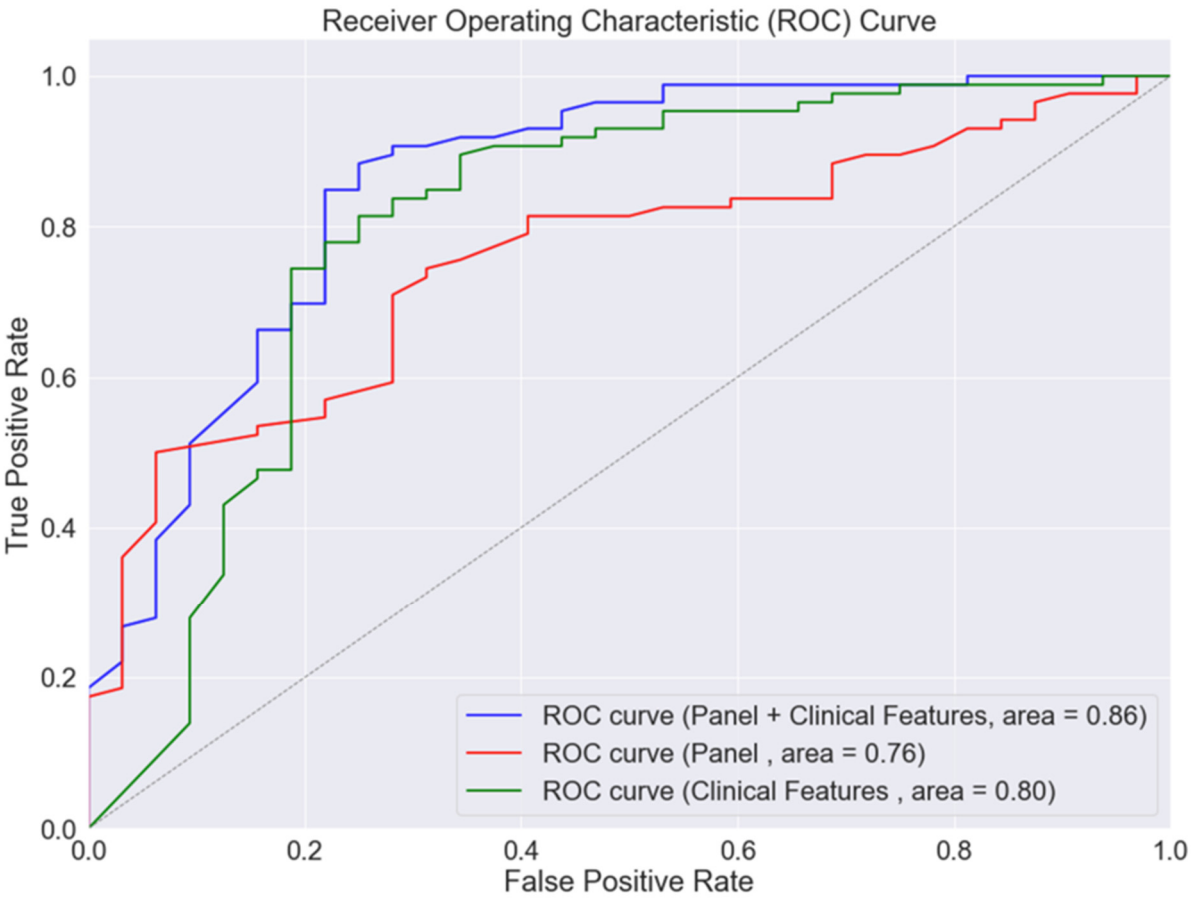

Figure 2. Random forest model performance for predicting limb loss at 2 years was evaluated using different input features: clinical characteristics alone (AUROC 0.80), the five-protein panel alone (AUROC 0.76), and clinical characteristics combined with the five-protein panel (AUROC 0.86). ROC: receiving operator characteristic, AUROC: area under the receiver operating characteristic.

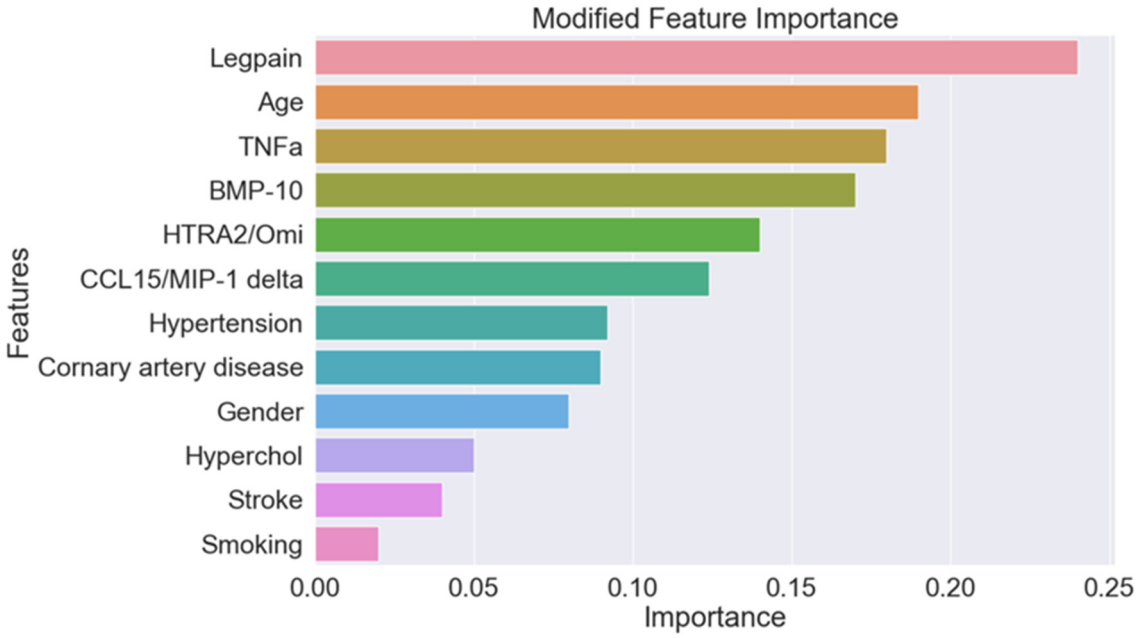

Figure 3. Importance of input features for predicting need for limb loss at 2 years with the random forest model. TNFa: tumor necrosis factor alpha, HTRA2/Omi: HTRA2, also known as Omi, CCL15/MIP1 delta: chemokine (c-c motif) ligand 15/macrophage inflammatory protein 1 delta.

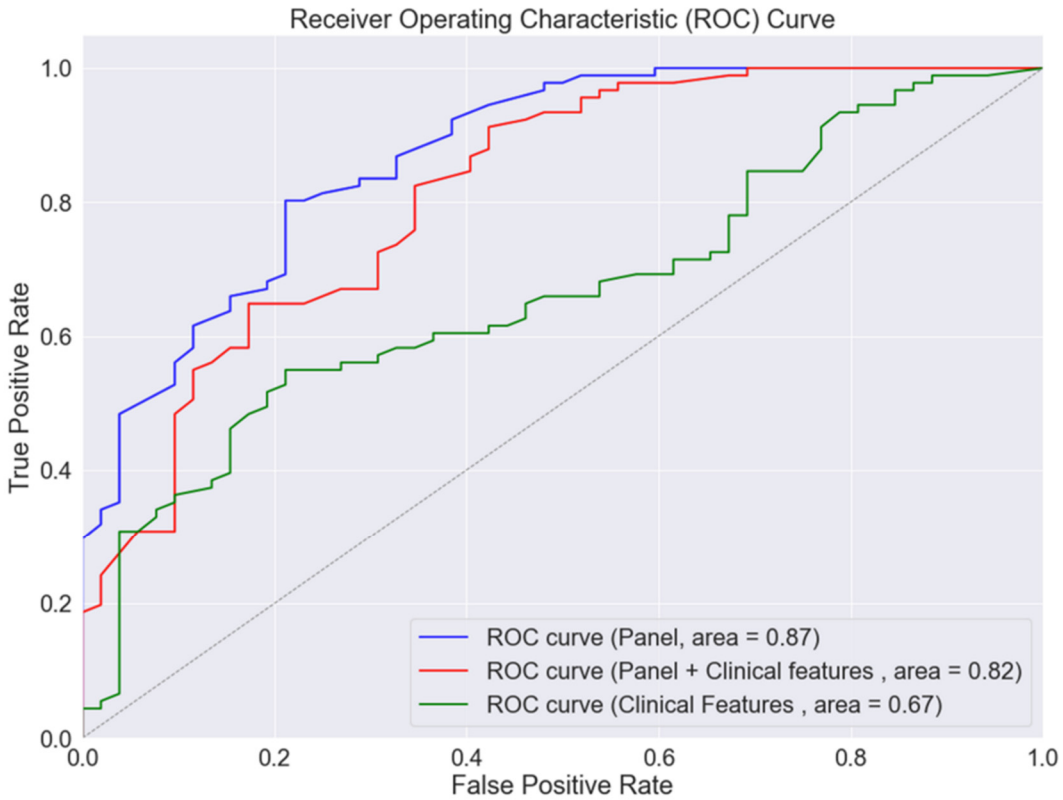

Figure 4. Random forest model performance for predicting need for vascular surgery at 2 years was evaluated using different input features: clinical characteristics alone (AUROC 0.67), the five-protein panel alone (AUROC 0.87), and clinical characteristics combined with the five-protein panel (AUROC 0.82). ROC: receiving operator characteristic, AUROC: area under the receiver operating characteristic.

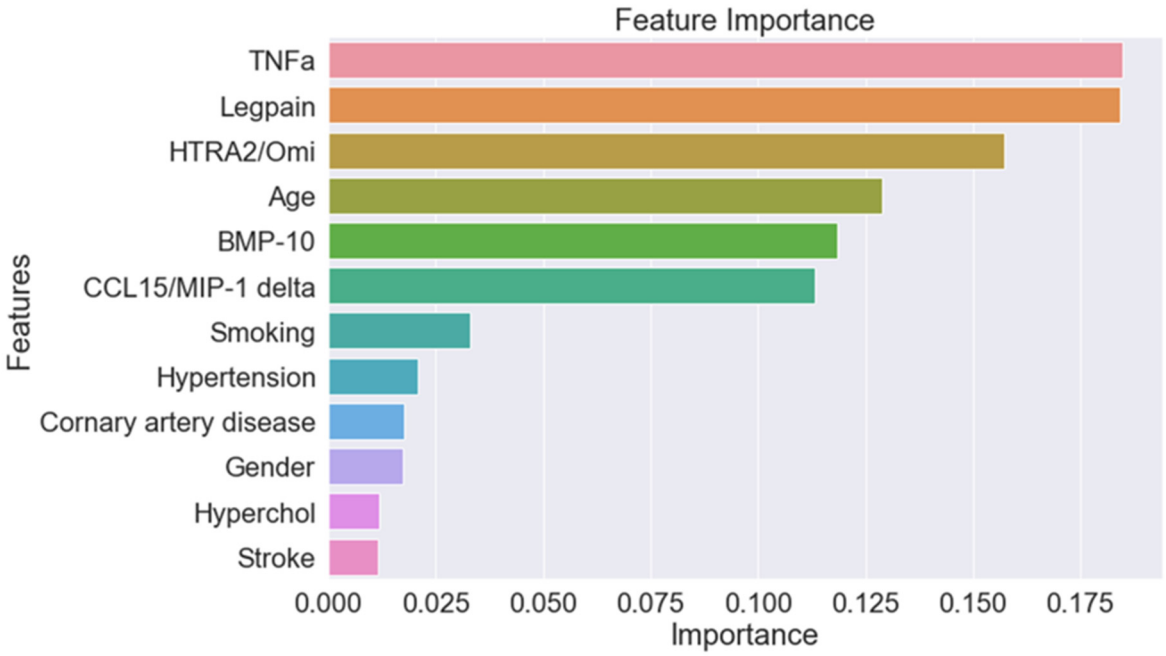

Figure 5. Importance of input features for predicting need for vascular surgery at 2 years with the random forest model. TNFa: tumor necrosis factor alpha, HTRA2/Omi: HTRA2, also known as Omi, CCL15/MIP1 delta: chemokine (c-c motif) ligand 15/macrophage inflammatory protein 1 delta.

3.6. Model Performance for Predicting Diabetic Foot Ulcers at 2 Years

The random forest model's performance for predicting diabetic foot ulcers at 2 years was evaluated using different input features: clinical characteristics alone (AUROC 0.60), the five-protein panel alone (AUROC 0.78), and clinical characteristics combined with the five-protein panel (AUROC 0.81). Incorporating the protein panel enhanced the model's predictive performance, resulting in an NRI of 0.13, and this was statistically significant (p = 0.0451) (Figure 6). The most significant protein for predicting diabetic foot ulcers at 2 years was TNFa (Figure 7).

3.7. Model Performance for Predicting MALE at 2 Years

The random forest model's performance in predicting MALE at 2 years was evaluated using different input features: clinical characteristics alone (AUROC 0.59), the five-protein panel alone (AUROC 0.87), and clinical characteristics combined with the five-protein panel (AUROC 0.86). Incorporating the protein panel significantly enhanced the model's predictive power compared to relying solely on clinical characteristics, resulting in an NRI of 0.59. This improvement was highly significant, with a p-value of less than 0.001 (Figure 8). Among the panel proteins, TNF alpha played the most significant role in predicting 2-year MALE in diabetic patients (Figure 9).

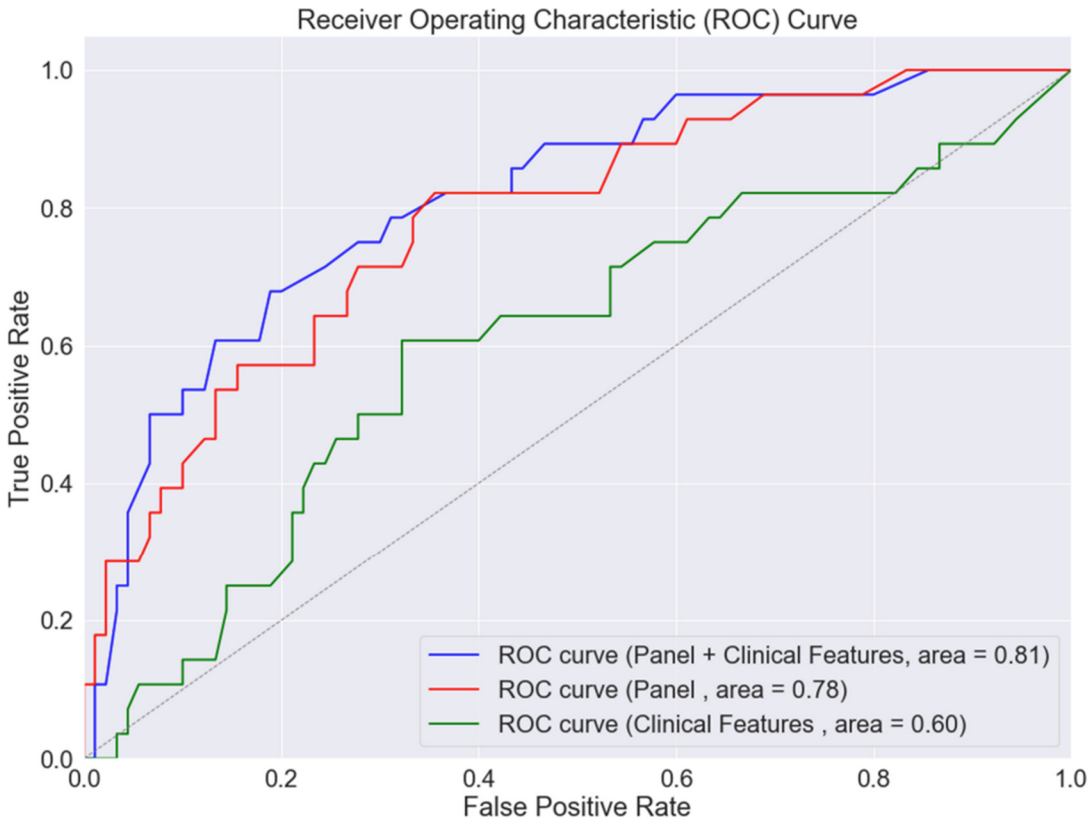

Figure 6. Random forest model performance for predicting diabetic foot ulcers at 2 years was evaluated using different input features: clinical characteristics alone (AUROC 0.60), the five-protein panel alone (AUROC 0.78), and clinical characteristics combined with the five-protein panel (AUROC 0.81). ROC: receiving operator characteristic, AUROC: area under the receiver operating characteristic.

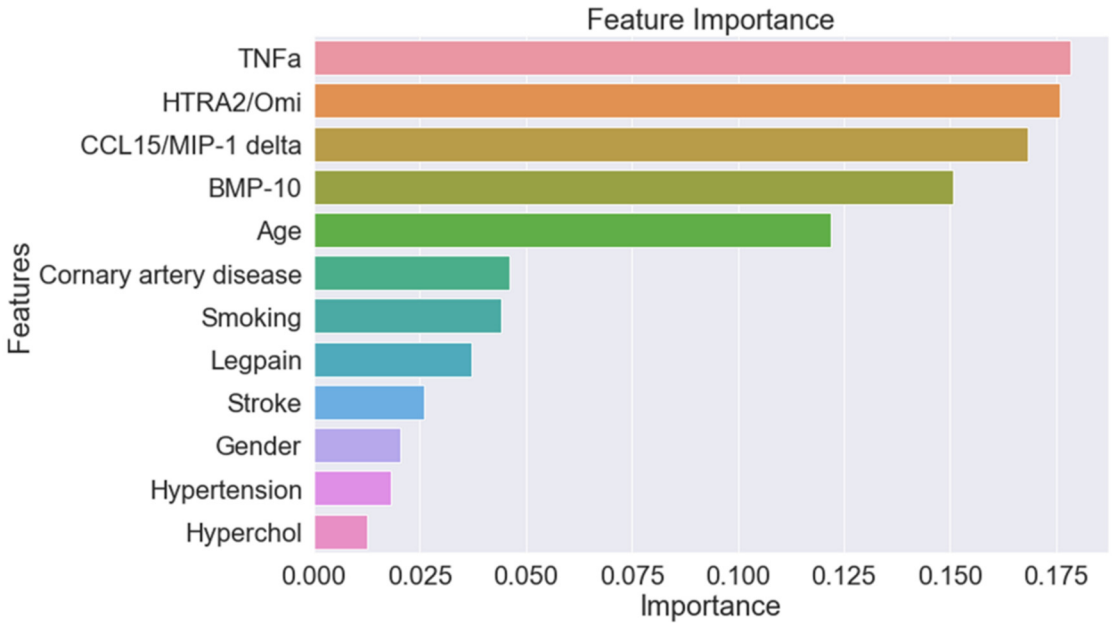

Figure 7. Importance of input features for predicting diabetic foot ulcers at 2 years with the random forest model. TNFa: tumor necrosis factor alpha, HTRA2/Omi: HTRA2, also known as Omi, CCL15/MIP1 delta: chemokine (c-c motif) ligand 15/macrophage inflammatory protein 1 delta, BMP-10: bone morphogenic protein 10.

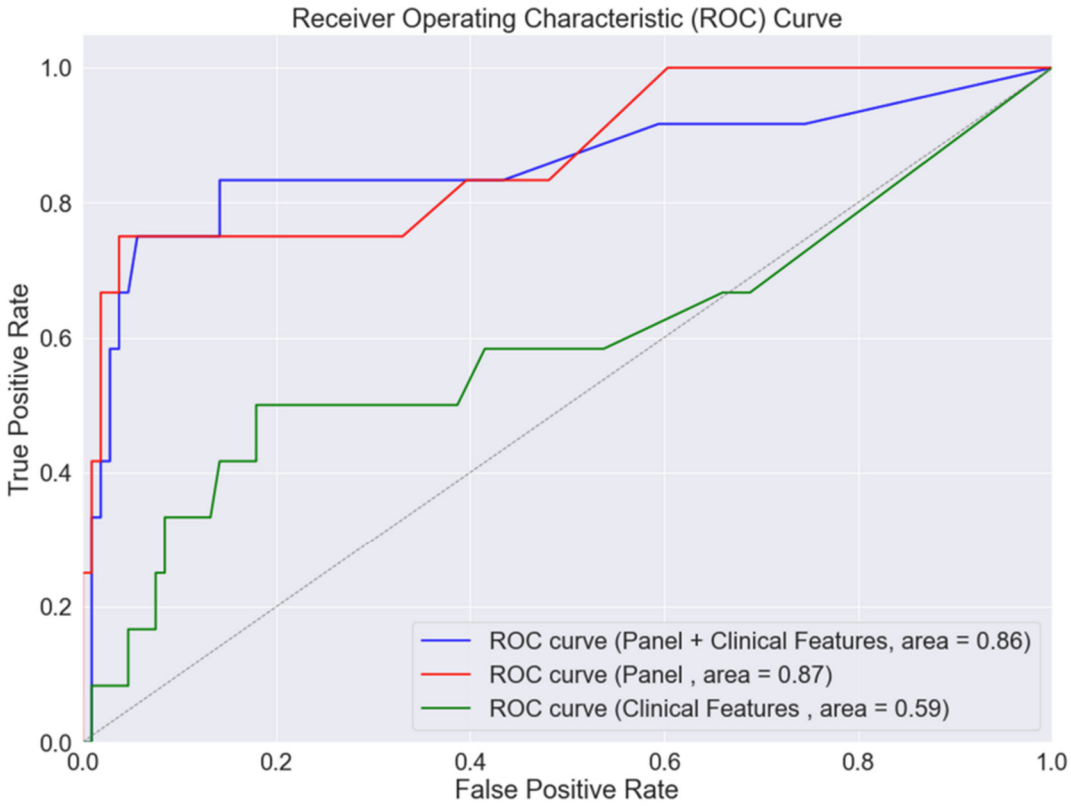

Figure 8. Random forest model performance for predicting MALE at 2 years was evaluated using different input features: clinical characteristics alone (AUROC 0.60), the five-protein panel alone (AUROC 0.78), and clinical characteristics combined with the five-protein panel (AUROC 0.81). ROC: receiving operator characteristic, AUROC: area under the receiver operating characteristic.

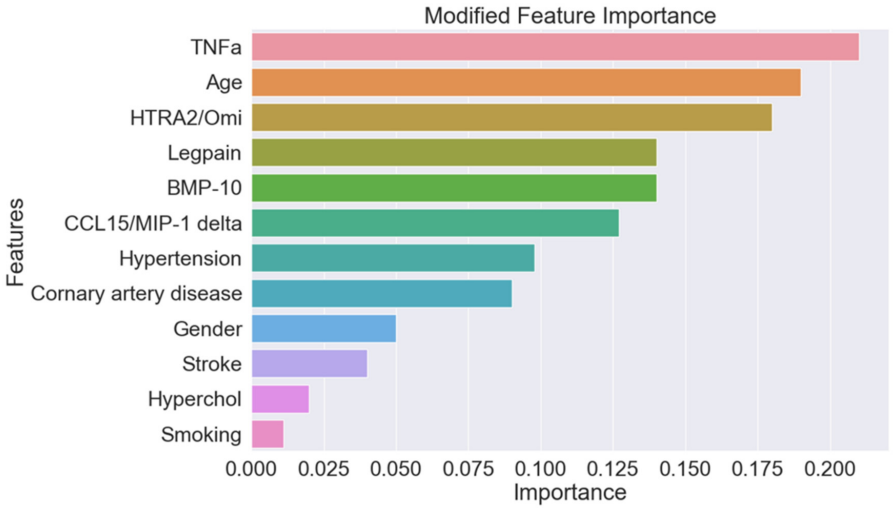

Figure 9. Importance of input features for predicting MALE at 2 years with the random forest model. TNFa: tumor necrosis factor alpha, HTRA2/Omi: HTRA2, also known as Omi, CCL15/MIP1 delta: chemokine (c-c motif) ligand 15/macrophage inflammatory protein 1 delta, BMP-10: bone morphogenic protein 10, MALE: major adverse limb event.

3.8. Kaplan–Meier Risk-Stratified Analysis

Each patient was assigned a specific score based on protein concentration, and the overall cohort was divided into high and low score groups. Kaplan–Meier analysis demonstrated that the high score group effectively stratified patients for MALE risk (p = 0.007; log rank = 7.18). MALE-free survival rates at 1 year and 2 years were 88% and 72%, respectively, in the low score group, and 57% and 41%, respectively, in the high score group (Figure 10).

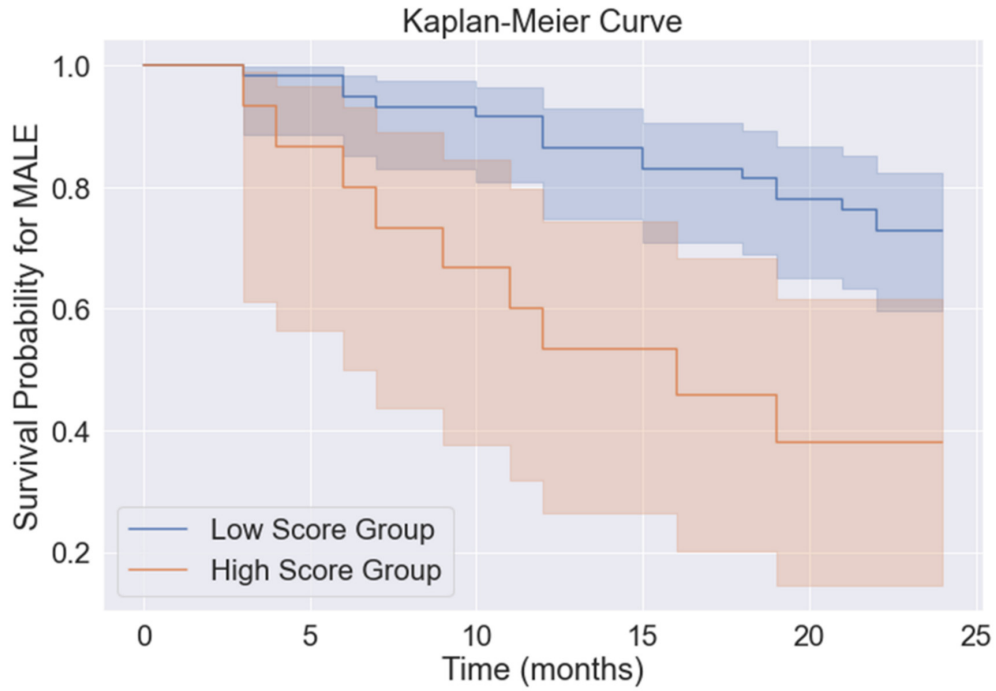

Figure 10. Kaplan–Meier analysis showing that the high score group effectively stratified patients for MALE risk (p = 0.007; log rank = 7.18). The MALE-free survival rates at 1 year and 2 years were 88% and 72%, respectively, in the low score group, and 57% and 41%, respectively, in the high score group. MALE: major adverse limb event, defined as the need for vascular intervention or major amputation at 2 years.

4. Discussion

4.1. Summary of Findings

In a cohort of 392 patients (DM non PAD 101, DM PAD 192) we identified five proteins (TNF alpha, BMP-10, CCL15/MIP-1 delta, HTRA2/Omi, MMP-10) that had a significantly higher level of expression in the DM PAD group. Combining the protein panel with clinical characteristics resulted in an NRI of 0.28 and statistically significant evidence of the ability to use this panel for the diagnosis of PAD (p = 0.027). TNFa contributed the most to predicting limb loss, need for vascular surgery, diabetic foot ulcer, and MALE. Using these five proteins for the panel, we were able to accurately predict MALE within 2 years using a random forest model. The accuracy of the model's predictions was increased when clinical characteristics were combined with the protein panel. In other words, clinical characteristics alone were suboptimal in predicting adverse limb events, and the addition of circulating biomarkers significantly improved the model's predictive performance, highlighting the value of these biomarkers in PAD prognosis.

4.2. Comparison with the Existing Literature–Biomarker Physiology

Multiple biomarkers in diabetic patients that link oxidative stress and endothelial dysfunction to cardiovascular disease and PAD have been identified; these biomarkers are associated with a prothrombotic state, increased inflammation, vascular cell proliferation, and/or disrupted vascular hemostasis [14–16,27]. The first protein from our panel, TNFa, is associated with vascular inflammation in patients with DM [27]. TNFa plays a crucial role in the pathophysiology of peripheral arterial disease (PAD) among diabetic patients through intricate biological pathways. This protein is primarily secreted by activated macrophages, Th1 cells, and natural killer cells; it triggers a cascade of inflammatory responses by binding to its receptors, TNFR1 and TNFR2 [28,29]. This interaction activates multiple signaling pathways, including the nuclear factor-kappa B (NF-κB) pathway, resulting in the upregulation of proinflammatory cytokines and adhesion molecules [30]. Chronic inflammation mediated by TNF-α promotes endothelial dysfunction, exacerbates oxidative stress, and induces smooth muscle cell proliferation, contributing to atherosclerosis progression [28–32]. Furthermore, TNF-α disrupts the balance of endothelial nitric oxide synthase (eNOS) activity, impairing vasodilation and promoting vasoconstriction [32]. These mechanisms collectively accelerate the development and progression of PAD in diabetic patients.

The next biomarker in our panel, BMP-10, has previously been studied in patients with atrial fibrillation, as it has atrial specificity and is associated with all-cause mortality, major adverse cardiac events, heart failure, and cardiovascular-related death [33–35]. In rats, hypertension and hyperglycemia induce BMP-10 expression, but the role of BMP-10 in humans with PAD and DM is not well studied [34,35]. BMP10, a member of the transforming growth factor-beta (TGF-β) superfamily, exerts its effects by binding to type I and type II serine/threonine kinase receptors, thereby activating downstream signaling pathways such as Smad1/5/8 [36,37]. Research on the specific role of BMP10 in diabetic PAD is still evolving, but it has potential as a therapeutic target to mitigate vascular complications in diabetic patients.

The third protein from our panel, CCL15/MIP-1, induces neovascularization and is elevated in diabetic nephropathy as well as in atherosclerotic plaques [38,39]. CCL15 belongs to the macrophage inflammatory protein-1 family of chemokines [40]. Upon binding to its cognate receptor, CCR1 or CCR3, CCL15 triggers downstream signaling cascades, including activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways, and leads to an inflammatory response [40]. However, its specific role in atherosclerotic plaque development and PAD progression in diabetes is not well understood.

The fourth protein, HTRA2/Omi, is a serine protease apoptosis-inducing protein [41]. It is stored in the mitochondria and plays a crucial role in maintaining mitochondrial homeostasis and regulating cellular apoptosis [41]. When apoptotic stimuli are present, HTRA2/OMI is released from the mitochondrial intermembrane space, and it potentiates apoptosis by inhibiting proteins that inhibit apoptosis, as well as by cleaving cytochrome C [41,42]. This cleavage leads to release of cytochrome c into the cytoplasm, activating the caspase cascade and initiating apoptotic cell death [41]. Additionally, HTRA2/Omi has been shown to modulate cellular stress responses and inflammatory pathways [43]. This protein has been shown to play a role in ischemia reperfusion injuries secondary to STEMI, as well as in the immune overactivation seen in neurodegenerative diseases and diabetes, but it is not well studied in PAD [42,43].

Lastly, MMP-10 is associated with poor outcomes in PAD, including cardiovascular events and mortality [44]. MMPs belong to a family of zinc-dependent endopeptidases and are first synthesized into an inactive pre-proMMP form [45,46]. During translation, a signal peptide is removed, and they become proMMPs; when cellular stressors activate the proMMPs they are converted to their active form, which goes on to break down proteins located in the extracellular matrix [45,46]. MMP10 is secreted by multiple cells, including vascular smooth muscle and vascular endothelial cells [45]. Specifically, MMP10 targets extra cellular matrix proteins, including collagen, elastin, fibronectin, and laminin [45]. MMPs are dysregulated in hyperglycemic states and are implicated in atherosclerosis, abdominal aortic aneurysms, tissue remodeling in limb ischemia, neointimal hyperplasia after intervention, and vascular complications of chronic kidney disease [44–48].

4.3. Comparison with the Existing Literature–Interplay Between Biomarkers, PAD, and DM

Hyperglycemia and inflammation are driving factors behind the expression of our panel proteins. It has been well established that chronic hyperglycemia induces epigenetic changes that lead to reactive oxygen species (ROS) production and vascular dysfunction [49–53]. Paneni et al. showed that even intermittent spikes in glucose can activate ROS, generating PKC and NADPH oxidase, as well as epigenetic changes that upregulate the NF-kB p65 transcription factor and inflammatory adhesion molecules [54]. Traditional markers of diabetic control such as hemoglobin A1C do not detect glycemic fluctuations, but rather an average over 3 months, so biomarkers involved in ROS generating mechanisms could offer an alternative measure to screen for PAD in diabetes [54,55].

4.4. Comparison with the Existing Literature–Random Forest Models and Biomarkers as Clinical Prediction Tools

Random forest models are generally high-performing and can provide accurate predictions in clinical settings [56]. Prediction models can help identify patients at risk of diabetic complications, predict outcomes, and improve screening, diagnosis, and treatment [11,57]. PAD and cardiovascular disease in patients with DM are usually asymptomatic in early stages of the disease [58,59], but they can have devastating outcomes if left uncontrolled [60]. The random forest model used in this study had multiple advantages that were achieved by combining decision trees, which resulted in reduced variance, the ability to interact with large data sets in an efficient matter, and minimization of overfitting [61]. Overall, our model, like other existing models, may be able to identify asymptomatic patients earlier and identify patients who may be missed with conventional testing when using biomarkers and clinical features as inputs [11,62,63].

4.5. Implications

This predictive model has the potential to guide clinical decision making in patients with PAD and DM. Firstly, this predictive model can aid in the screening and diagnosis

of patients with diabetes and PAD. These patients are at higher risk for adverse outcomes, including limb loss, foot ulcers, and MALE [6–8]. Secondly, patients who are identified as being at increased risk can have preventative measures implemented, including patient education, duplex ultrasound to assess PAD, referral to an endocrinologist for better glycemic control, referral to chiropody for foot care and offloading, and referral to a vascular surgeon for PAD management [64]. Using this predictive model in combination with physician expertise, we will be better equipped to take a proactive approach rather than a reactive approach, which has potential to improve patient outcomes, reduce health care costs, and increase limb salvage [65].

4.6. Limitations

This study has multiple limitations. First, this study recruited patients from a single center and excluded patients with certain diseases (acute limb ischemia, acute coronary syndrome, elevated troponin, or having taken biological anti-inflammatory medications in the last 3 months). Both of these factors may decrease the generalizability of the results. Excluding patients with the aforementioned conditions may have created selection bias. The rationale for excluding these patients was to focus on patients with chronic diabetes and PAD, as well as to remove patients with conditions known to drastically elevate specific biomarkers such as creatinine kinase in acute limb ischemia and troponin in acute coronary syndrome. Second, the outcomes were reported from a 2-year follow-up; a longer follow-up period could provide more information regarding the prognostic capabilities of the protein panel. Third, we used a relatively small cohort to train the random forest model, and having a larger sample to train from may increase the model's performance. Fourth, this study focused on limb-related outcomes; however, given the interplay among vascular, cardiac, and cerebrovascular pathology in patients with DM and PAD, future studies could also assess cardiac and cerebrovascular events.

5. Conclusions

This study identified five proteins (TNF alpha, BMP-10, CCL15/MIP-1 delta, HTRA2/Omi, and MMP-10) that are expressed at higher levels in patients with both diabetes and peripheral artery disease compared to patients with diabetes but no PAD. We demonstrated that these proteins can be combined with clinical characteristics as inputs for our random forest model to accurately diagnose peripheral artery disease as well as to predict major adverse limb events, the need for vascular surgery, limb loss, and diabetic foot ulcers over a 2-year period. These biomarkers have the potential to aid in screening for PAD in patients with diabetes. Larger studies with longer follow-up periods are needed to corroborate the findings of this study.

Author Contributions: K.D.: interpretation of data, drafting, and revision. B.L.: interpretation of data, drafting, and revision. M.S.: interpretation of data, drafting and revision. F.S.: interpretation of data, drafting, and revision. A.Z.: acquisition, analysis, and interpretation of data. B.J.A.: interpretation of data, drafting, and revision. K.R.: interpretation of data, drafting, and revision. H.K.Y.: interpretation of data, drafting, and revision. R.A.: interpretation of data, drafting, and revision. M.Q.: conception, design of work, acquisition, analysis, interpretation of data, and revision. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded through support from the Blair Foundation. The funder did not play a role in the design or conduct of this research.

Institutional Review Board Statement: This study was granted approval by the research ethics board at Unity Health Toronto, University of Toronto, Canada. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Unity Health Toronto, University of Toronto, Canada, on 8 February 2017 (REB # 16-365). All patients provided informed consent to participate in this study.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

ABI	ankle brachial index
ACE-i	angiotensin-converting enzyme inhibitor
ARB	angiotensin receptor blockers
B-Bl	beta blocker
CCB	calcium channel blocker
ASA	acetylsalicylic acid
TNFa	tumor necrosis factor alpha
BMP-10	bone morphogenic protein 10
CCL15/MIP1 delta	chemokine (c-c motif) ligand 15/macrophage inflammatory protein 1 delta
MMP-10	matrix metalloprotease 10
HTRA2/Omi	HtrA serine peptidase 2
AUROC	area under the receiver operating characteristic curve
DM	Diabetes mellitus
eNOS	endothelial nitric oxide synthase
HCTZ	hydrochlorothiazide
MALE	major adverse limb events
MAPK	mitogen-activated protein kinase
NF-κB	Nuclear factor-kappa B
NRI	net reclassification improvement
PAD	peripheral arterial disease
ROS	reactive oxygen species
TGF-β	transforming growth factor-beta

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