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Proteomic analysis of carotid artery plaques with and without vulnerable features on magnetic resonance angiography with vessel wall imaging: a pilot study

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ABSTRACT

Objective: Extracranial carotid artery pathology accounts for 15% to 20% of ischemic strokes. Advancements in magnetic resonance angiography (MRA) with vessel wall imaging (VWI) have enabled the identification of vulnerable plaques, aiding in risk stratification for neurovascular events. This pilot study aimed to identify proteins in plaques with and without vulnerable features on MRA with VWI.

Methods: Consecutive patients undergoing carotid endarterectomy were included in the study cohort with preoperative MRA with VWI. A retrospective chart review was conducted to extract pertinent clinical data including cardiovascular risk factors and medications. Proteomic analysis involved Tandem Mass Tag (TMTpro) labeling of peptides, basic pH high-performance liquid chromatography fractionation, and NanoLC-tandem mass spectrometry.

Results: Proteomic analysis revealed 23 proteins significantly elevated in vulnerable plaques, including Proteinase 3 (PRTN3), Phospholipid Transfer Protein (PLTP), and S100 Calcium-Binding Protein A12 (S100A12), with increased abundance exceeding two-fold changes or above ($P < .001$). Conversely, three proteins exhibited reduced abundance in vulnerable plaques including Dynamin-3 (DNM3), Transmembrane Protein 181 (TMEM181), and Adducin-3 (ADD3) ($P < .05$).

Conclusions: This study contributes to the understanding of protein biomarkers associated with carotid plaque vulnerability, offering insights into disease progression and stroke prevention. Proteins secreted by vulnerable plaques may offer not only the potential for early disease recognition; but can also become a target for future pharmacologic therapy prior to a devastating neurologic event. Further validation studies and multi-center trials will be needed to confirm the value of these potential biomarkers. (JVS—Vascular Science 2025;6:100281.)

Clinical Relevance: Vulnerable plaques express unique protein signatures that may place individuals at a higher risk for atherosclerotic neurovascular disease. These proteins may serve as biomarkers for early recognition and surgical/pharmacologic intervention in patients with high risk for stroke associated with vulnerable plaques.

Keywords: Carotid artery endarterectomy; Magnetic resonance angiography with vessel wall imaging; Proteomics

Extracranial carotid artery stenosis is responsible for 15% to 20% of ischemic strokes.[1,2] Recently, magnetic resonance angiography (MRA) with vessel wall imaging (VWI) has been utilized to characterize vulnerable plaques by recognizing features such as intraplaque hemorrhage, ulcerated fibrous cap, and lipid rich necrotic core, which are deemed at a higher risk for neurovascular events.[3-5]

Several studies have been published on the use of biomarkers to identify atherosclerotic plaque vulnerability; however, results are preliminary. Moreover, there is no consensus on source of investigational material (ie,

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Additional material for this article may be found online at www.jvsvs.org.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the Journal policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

2666-3503

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https://doi.org/10.1016/j.jvssci.2025.100281

plaque, serum, and/or plasma), and the analytical methods/techniques to pursue proteomic studies are also diverse.[6] Therefore, our interest in conducting a systematic pilot study identifying on cross-sectional imaging of vulnerable plaques. These plaques were then analyzed for proteins enriched using spectrometry that can serve as biomarkers of at-risk atherosclerotic plaques.

ARTICLE HIGHLIGHTS

- **Type of Research:** Single-center cohort study
- **Key Findings:** Proteomic analysis of carotid plaques with and without vulnerable features identified on magnetic resonance angiography with vessel wall imaging have differences in protein composition. A differential proteomic profile between vulnerable and atherosclerotic plaques was evident, with signature proteins offering insights regarding disease progression.
- **Take Home Message:** Vulnerable plaques express unique protein signatures that may place individuals at a higher risk for atherosclerotic neurovascular disease. Further studies may be needed to validate and assess the role of these proteins as potential biomarkers in patients with high risk for stroke associated with vulnerable plaque.

METHODS

A retrospective review of consecutive patients who presented to our vascular surgery clinic from April 2019 to May 2021 was performed. We focused on those individuals with cross-sectional imaging in the form of MRA with VWI with and without features of plaque vulnerability. These were grouped into vulnerable (VP) and atherosclerotic plaque (AP). After plaque removal at the time of endarterectomy, these then underwent proteomic analysis using mass spectrometry. Additionally, a comprehensive chart review was conducted to extract pertinent clinical information for each patient, including patient demographics, past medical history, medications, and relevant laboratory data. This study was approved by the Mayo Clinic Institution Review Board (IRB# 19-007,071). Written informed consent was not obtained for the study because it was deemed minimal risk to the patients.

Vulnerable plaque definition and MRA protocol. All patients underwent a 3.0 Tesla MRA with VWI. Plaque morphology was evaluated by a blinded board-certified neuroradiologist as part of the preoperative workup of the patient. The presence of vulnerable features was assessed and identified as intraplaque hemorrhage, plaque ulceration, plaque inflammation, and/or lipid necrotic core.[5]

Tandem Mass Tag labeling of peptides. Approximately 70 ug of dried peptide from each sample was solubilized in 90 uL of 100 mM triethylammonium bicarbonate, pH 8.5/10% acetonitrile, and mixed with 500 ug of a unique Tandem Mass Tag (TMTpro) reagent solubilized in 10 uL of acetonitrile. After incubation for 1 hour at room temperature, the reactions were quenched with 5 uL of 5% hydroxylamine, and a 2-uL aliquot from each sample was pooled and analyzed by tandem mass spectrometry (MS/MS) to ensure the labeling efficiency was ≥98%. The samples were then pooled to match the reporter ion intensities from each channel and the mix diluted to 4 mL in 0.1% TFA/5% acetonitrile. Excess TMT reagents were removed using solid phase extraction with a Waters Sep Pak Plus C18 cartridge, and the eluted TMTpro labeled peptides were lyophilized.

Basic pH high-performance liquid chromatography fractionation. To reduce the sample complexity, the dried peptide mixture was solubilized in 500 uL 5 mM ammonium formate, pH 8.5, and separated into 96 fractions using a Dionex Ultimate 3000 RS high-performance liquid chromatography system with a Waters XBridge BEH C18 4.6 mm × 250 mm column. The system was set up with 5 mM ammonium formate; pH 8.5 in water for the A solvent and 5 mM ammonium formate; pH 8.5/90% acetonitrile for the B solvent. The separation gradient was 5% B to 60% B over 60 minutes followed by a 2-minute jump to 80% B while maintaining a constant flow rate of 0.5 mL/minute. The 96 fractions were concatenated to 12 fractions and lyophilized.

NanoLC-tandem mass spectrometry data acquisition. The peptide fractions were analyzed by nanoLC-MS/MS using a Thermo Scientific Exploris 480 Orbitrap mass spectrometer coupled to a Thermo Ulimate 3000 RSLCnano HLPC system with 0.1% formic acid in 98% water/2% acetonitrile for the A solvent and 0.1% formic acid in 80% acetonitrile/10% isopropanol/10% water for the B solvent. Each fraction was solubilized in 0.1% formic acid and pumped onto a Halo C18 2.7 μm EXP stem trap (Optimize Technologies) with 0.1% formic acid/0.05% TFA at a flow rate of 8 mL/min. The trap was placed in line with a 50 cm × 75 um EasySpray C18 nanocolumn running a flow rate of 300 nL/min, and the peptides were separated with a gradient of 5% B solvent to 35% B solvent over 100 minutes, then a 14-minute jump to 90% B then a 4-minute hold. The Orbitrap Exploris 480 was set for data dependent acquisition with a 3-second cycle. The MS1 survey scan range was from 350 to 1400 m/z at resolution 120,000 (at 200 m/z) with the AGC set for a maximum of 1×10^6 ions and a 50 ms ion injection time.

Ions with positive charge states from 2 to 5 were sequentially selected for high-energy collisional dissociation fragmentation MS/MS scans in the orbitrap at

resolution 45,000 with a normalized collision energy setting of 32 and the isolation width set to 0.7 m/z. The MS2 AGC setting was 200% (2×10^5 ions), and the max ion injection time was set to auto. The dynamic exclusion feature was used to prevent the ions already used for MS/MS scans and any ions within ± 7 ppm from being selected again for fragmentation for at least 30 seconds.

Protein identification and TMTpro quantitation. The mass spectrometry raw data files were searched with Andromeda against the Swissprot human database (ver. 2021_04) in MaxQuant (ver1.6.17), setup for MS2 reporter ion quantification with TMTpro 16plex isobaric labels. Parameters were set for full trypsin specificity with oxidized Met and N-term protein acetylation allowed as variable modifications and carbamidomethyl cysteine as a fixed modification. Mass tolerances were set at 4.5 ppm for MS1 and 20 ppm for MS2, and protein identifications with a 1 peptide minimum were filtered at 1% false discover ratae (FDR) at the peptide and protein level. Protein TMT quantitation was performed using an inhouse R-script to calculate fold-change and *P*-values. Reporter ion channel correction factors were applied to peptide spectral matches, and peptides were removed that exceeded the threshold maximum of 50% isolation interference. Sample groups missing 50% of values were removed for comparisons with no imputation applied. Sample normalization was by median subtraction, and *t*-test comparisons were made with protein level log2 ratios. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with dataset identifier PXD052120 and 10.6019/PXD052120.[7]

EXPERIMENTAL PROCEDURES

Formalin-fixed paraffin-embedded tissue collection. Formalin-fixed paraffin-embedded (FFPE) sections of 10 μm from the carotid artery plaque region were obtained from 16 patients, and these were prepared as follows.

FFPE sample processing and protein digestion. Five FFPE tissue scrolls of 10-μm thickness collected in 2 mL microfuge tubes were used for each plaque case. Tubes were heated at 65 °C for 30 minutes to melt the paraffin. While still warm, 1 mL of xylene was added to each tube, and tubes were rotated for 30 minutes at room temperature. Tubes were centrifuged at 17k × g for 10 minutes, and supernatant was discarded. This step was repeated once. A series of ethanol washes was done from 100% ethanol to 70% ethanol, then water, with tubes centrifuged and supernatant discarded at each step.

For protein extraction and digest, 200 μL of 5% sodium dodecyl sulfate in 50 mM triethylammonium bicarbonate buffer and 250 mM Tris, pH 8.2, was added to each tube, and samples were heated at 98 °C for 1 hour with mixing at 800 rpm (ThermoMixer C, Thermofisher Scientific). Sample tubes were loaded on a BioRuptor Pico for 20 cycles of 30 seconds ultrasonication on, 30 seconds off at 4 °C. Tubes were subjected to a second heating step at 98 °C for 1 hour. After centrifugation at 17k × g for 10 minutes, protein concentration of the supernatants was determined by the BCA protein assay (Thermofisher Scientific).

The same amount of protein from each sample was prepared for digestion and desalting on the mini S-Trap (Protifi) according to product instructions. Briefly, proteins were reduced and alkylated, acidified, exposed to high concentration of methanol, and trapped on the spin column. After washing, proteins are digested overnight with Trypsin/LysC (Promega Biotech) on the column, and desalted peptides are eluted with digest buffer followed by 0.1% trifluoroacetic acid (TFA), followed by 50% acetonitrile and 0.1% TFA.

RESULTS

Patient demographics. Fifteen patients underwent carotid endarterectomy (CEA) for a total of 16 carotid plaque specimens for proteomic analysis. Baseline characteristics of this cohort of patients are summarized in Supplementary Table I (online only). Mean age at the time of the CEA was 69.9 years (standard deviation [SD], 8.0 years). There were 73.3% male patients. The mean body mass index was 28.3 kg/m^2 (SD, 5.6 kg/m^2). Comorbidities included hyperlipidemia in 93.3%, hypertension in 80.0%, diabetes mellitus in 40.0%, coronary artery disease in 26.7%, peripheral arterial disease in 13.3%, and atrial fibrillation in 6.7% of patients. Preoperative medications included aspirin in 100.0%, clopidogrel in 37.5%, coumadin in 6.3%, and rivaroxaban in 6.3% of patients, respectively. Lipid-lowering medications were prescribed in 93.8% and antihypertensive medications in 68.8% of patients.

Of all carotid plaque lesions, most were left-sided (75%). Eleven plaques were found to have at least one vulnerable feature on MRA with VWI. From those, six of 11 had intraplaque hemorrhage, one of 11 had an ulcerated plaque, and one of 11 had an inflammatory plaque. Further, one of 11 had intraplaque hemorrhage and inflammation, one of 11 had intraplaque hemorrhage and ulceration, and one of 11 had intraplaque hemorrhage, ulceration, and inflammation. Mean degree of stenosis on MRA was 68.12% ± 17.07% (median, 70%; interquartile range, 60%-72.5%) (Table).

There were 31.3% (5/16) plaques from symptomatic patients: four of five with transient ischemic attacks (TIAs) and one of five with a stroke. Four of the five symptomatic patients had vulnerable features on MRA. Specifically, three patients with previous TIA had intraplaque hemorrhage (1/3), inflammatory plaque (1/3), and intraplaque hemorrhage with ulceration (1/3). The patient who had a stroke had the presence of both intraplaque hemorrhage and an inflammatory plaque.

Table. Summary of proteins from proteomic analysis with significant abundance in plaque from carotid endarterectomy (CEA)

Protein Name	Gene	Fold change VP/AP	Ratio VP/AP	*P*-value	*P*-value FDR corrected	Correlation to atherosclerosis	Main function
Proteinase 3	PRTN3	3.64	3.64	<.001	.03	Proapoptotic activity, and inflammatory properties with cytokines release.	Neutrophil transendothelial migration and protease properties.
Phospholipid Transfer Protein	PLTP	3.59	3.59	<.001	.01	Inflammation, microcalcification, and increased expression of osteopontin (marker of plaque instability)	Mediates transfer of lipids and cholesterol in between lipoproteins.
S100 Calcium-Binding Protein A12 (Calgranulin C)	S100A12	3.21	3.21	<.001	.03	Plaque progression, not known mechanism.	Leukocytes recruitment and chemokine production.
Elastase, Neutrophil Expressed	ELANE	3.20	3.20	<.001	.03	Endothelial dysfunction, leukocyte recruitment, foam cell formation, and plaque destabilization	Regulates NK, monocyte, and granulocytes functions and inhibits indirectly pyroptosis.
Apolipoprotein M	APOM	2.55	2.55	<.001	.01	Not known correlation.	Lipid transport regulation.
Cathelicidin Antimicrobial Peptide 1	CAMP	2.50	2.50	<.001	.02	Inflammatory and immunological effects by T cell activation.	Cytoskeleton regulation.
Paraoxonase 1	PON1	2.49	2.49	<.001	.01	Anti-atherogenic properties by retarding LDL and cholesterol oxidation and promoting HDL antioxidant effects.	Hydrolase associated to LDL protection against oxidative modification.
Granulin	GRN	2.45	2.45	<.001	.04	Inflammation through signaling with IL-8.	Regulation of lysosomal function, inflammation, wound healing, and cell proliferation.
Apolipoprotein D	APOD	2.33	2.33	<.001	.01	Not known correlation.	Part of the lecithin-cholesterol acyltransferase, probably associated to bilin transport.
Apolipoprotein L1	APOL1	2.26	2.26	<.001	.02	Not known correlation.	Probable role in lipid exchange and transport.
Beta-2-glycoprotein 1	APOH	2.23	2.23	<.001	.03	Inflammatory and autoimmune mediation for plaque progression, not known mechanism.	Probable prevention in activation of the intrinsic blood coagulation cascade.

Table. Continued.

Protein Name	Gene	Fold change VP/AP	Ratio VP/AP	*P*-value	*P*-value FDR corrected	Correlation to atherosclerosis	Main function
Lactotransferrin (Lactoferrin)	LTF	2.23	2.23	<.001	.04	Protective effects by antioxidant, anti-inflammatory and cholesterol regulating properties in plaques.	Iron binding transport protein.
Complement C1r Subcomponent-Like Protein	C1RL	2.21	2.21	<.001	.01	Not known correlation.	Proteolytic cleavage of HP/haptoglobins in the endoplasmic reticulum. Not associated yet to C1 complement cleavage.
Apolipoprotein B	APOB	2.21	2.21	<.001	.01	Not known correlation.	Major protein in chylomicrons, LDL and VLDL.
Solute Carrier Family 2 Member 14 (GLUT14)	SLC2A14	2.19	2.19	<.001	.01	Not known correlation.	Hexose transporter.
Ferritin Light Chain	FTL	2.19	2.19	.016	.09	Inflammation perpetuation in the plaque, not known mechanism.	Iron storage and homeostasis.
Insulin-Like Growth Factor Binding Protein Acid Labile Subunit	IGFALS	2.18	2.18	<.001	.02	Not known correlation.	Mediated protein-protein interactions in protein complexes, receptor-ligand binding, or cell adhesion.
Defensin Alpha 1	DEFA1	2.12	2.12	.01	.06	Not known correlation.	Induces production of pr-inflammatory cytokines and antibiotic properties.
Band 3 anion transport protein	SLC4A1	2.09	2.09	<.001	.04	Not known correlation.	Transporter for electroneutral anion exchange.
Peptidoglycan recognition protein 2	PGLYRP2	2.09	2.09	<.001	.04	Pro-atherogenic lipid profile and inflammation, not known mechanism.	Bacterial recognition protein.
Plasma Kallikrein (Kallikrein B1)	KLKB1; GZMH	2.07	2.07	<.001	.01	Plaque progression and inflammation, not known mechanism.	Surface dependent activation of the blood cascade.
Phospholipase D1 (Autotaxin)	GPLD1	2.02	2.02	<.001	.04	Indirect association by increasing LPA, which leads to production of inflammatory cytokines, platelet aggregation and chemotaxis, not known mechanism.	Involved in signal transduction, membrane trafficking, and the regulation of mitosis.
S100 Calcium-Binding Protein A8 (Calgranulin A)	S100A8	2.02	2.02	<.001	.03	Plaque progression, not known mechanism.	Induce neutrophil chemotaxis and adhesion.

(Continued on next page)

Table. Continued.

Protein Name	Gene	Fold change VP/AP	Ratio VP/AP	*P*-value	*P*-value FDR corrected	Correlation to atherosclerosis	Main function
Dynamin-3	DNM3	−2.00	0.50	.02	.09	Not known correlation.	Microtubule production and vesicle endocytosis.
Transmembrane Protein 181	TMEM181	−2.06	0.48	.04	.15	Not known correlation.	Mediates action of cytolethal distending proteins from bacteria.
Adducin-3	ADD3	−3.34	0.30	.02	.11	Plaque progression, not known mechanism.	Cytoskeleton protein of the spectrin-actin network.

AP, Atherosclerotic plaque; *FDR*, false discovery rate; *HDL*, high-density lipoprotein; *IL*, interleukin; *LDL*, low-density lipoprotein; *VP*, vulnerable plaque.

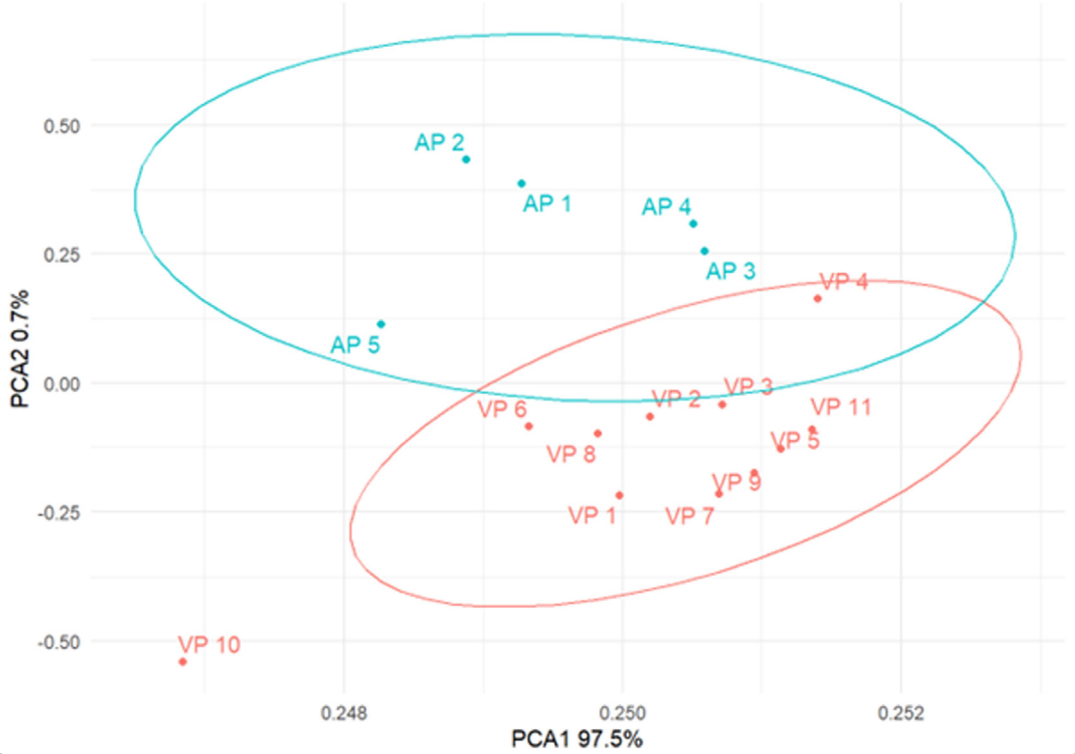

Fig 1. Principal component analysis (*PCA*) demonstrating sample clustering of plaque specimens between vulnerable plaque (*VP*) and atherosclerotic plaque (*AP*) groups. *Blue cluster*, AP; *red cluster*, VP.

Most plaques (83.3%) had less than 50% circumferential calcification on cross-sectional imaging. From the surgical standpoint, there was one superficial wound infection that required washout 21 days after CEA. Over a follow-up period averaging 802.13 days (SD, 509 days), there were two reported deaths of unknown causes at postoperative days 600 and 1319, respectively. There were no postoperative or follow-up strokes or TIAs reported.

Plaque proteomics. A total of 23 proteins from 2072 studied proteins were found to be present in at least two-fold higher in the VP group in comparison to the AP group. Our principal component analysis (PCA) demonstrates a sample clustering of high concordance according to cross-sectional imaging grouping data (see Fig 1). Proteinase 3 (PRTN3), Phospholipid Transfer Protein (PLTP), S100 Calcium-Binding Protein A12 (S100A12), and Elastase (ELANE) displayed notable higher proportions with ratios of 3.64, 3.59, 3.21, and 3.20, respectively, all with significant *P*-values ($< .001$). For a depiction of the heat map and volcano plot of all relevant proteins, please refer to Figs 2 and 3. Similarly, Apolipoprotein M (APOM),

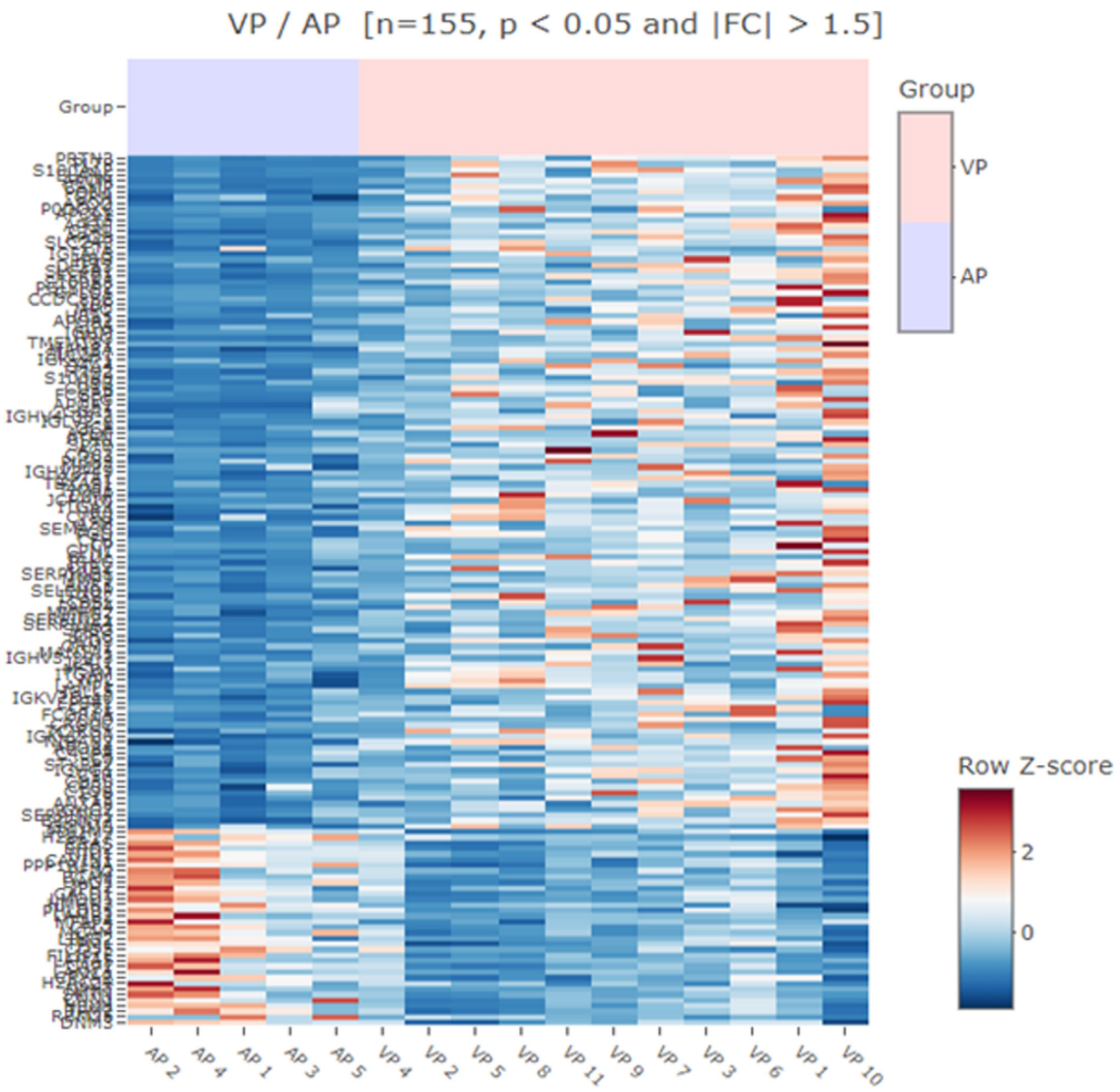

Fig 2. Heatmap analysis of all relevant proteins identified among patients with vulnerable plaque (*VP*) and atherosclerotic plaque (*AP*).

Cathelicidin Antimicrobial Peptide (CAMP), and Paraoxonase 1 (PON1) were present in higher proportions with ratios of 2.55, 2.50, and 2.49, accompanied by significant *P*-values (< .001). The complete list of proteins and respective values can be found in the Table.

Dynamin-3 (DNM3), Transmembrane Protein 181 (TMEM181), and Adducin-3 (ADD3) demonstrated at least two-fold higher prevalence in the AP group in comparison to the VP group. DNM3 exhibited a −2.00, TMEM181 a −2.06, and ADD3 a −3.34. Their *P*-values remained low, ranging from .02 to .04. However, when adjusted for FDR, only TMEM181 retained significance, with an FDR corrected *P*-value of .15.

DISCUSSION

Atherosclerosis is a complex disease process with multiple underlying molecular mechanisms involved. Studies conducted on protein biomarkers for atherosclerotic carotid plaque instability have been summarized in our previous publication.[6] Otherwise, most published studies are in animal models or in human coronary or aortic atherosclerotic plaques, and the role of these proteins in carotid atherosclerotic disease and stroke events are rarely reported.[8-10] Our pilot study has been able to elucidate 28 potential protein biomarkers for stability/instability of carotid artery plaques. Of these, 15 have demonstrated relevance in the pathogenesis of atherosclerotic disease.

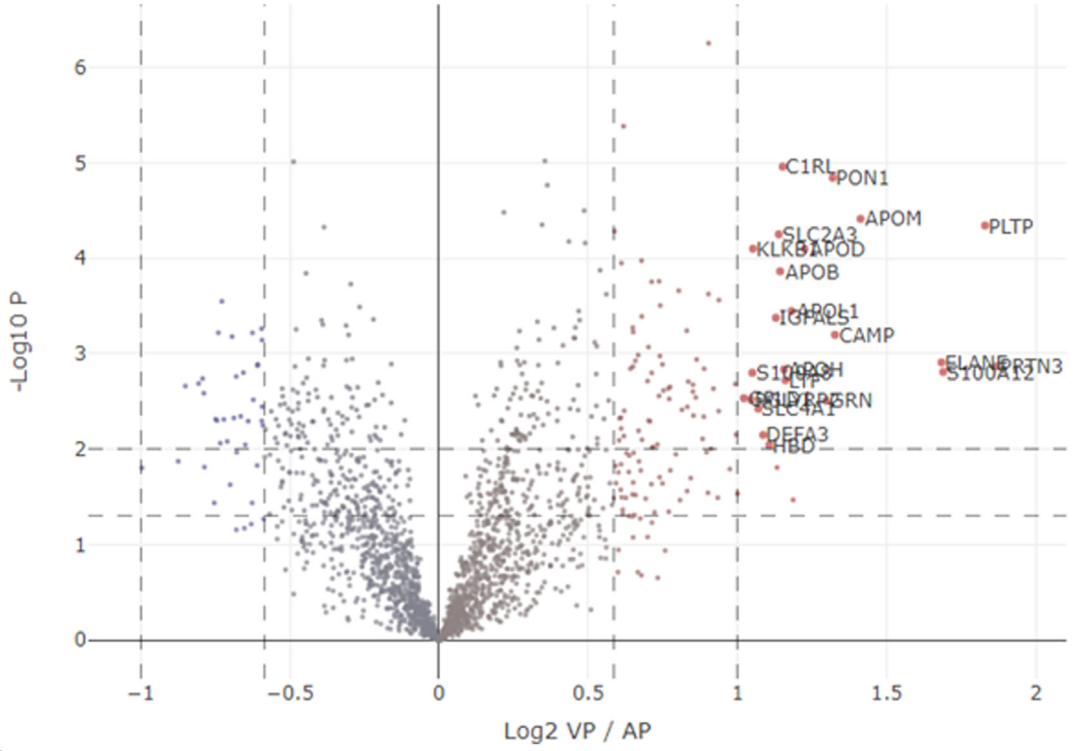

Fig 3. Volcano plot showing the differentially expressed proteins among vulnerable plaque (*VP*) and atherosclerotic plaque (*AP*) groups; upregulated proteins are shown in the right side of the plot.

Proteins involved in lipid metabolism. Apolipoproteins play a crucial role in lipid metabolism. Specifically, apolipoprotein A F2 fragments have shown an increased concentration in ruptured plaques in carotid disease, leading to a probable association of F2 fragments concentration and plaque instability.[11] A second study demonstrated that apolipoprotein A1 has higher expression in ruptured carotid plaques compared with non-ruptured carotid plaques.[12] In our study, the following apolipoproteins were found in greater than two-fold abundance: APOB, APOD, APOL1, and APOLM ($P < .001$), which may as well be associated with plaque ulceration.

PLTP is a mediator of lipid transfer to lipoproteins. In our study, PLTP was found in 3.59 times abundance in the VP group. In a previous study in diabetic patients, elevated plasma PLTP activity was associated with increased carotid artery intima-media thickness, a well-known marker of atherosclerosis, suggesting its important role in plaque formation.[13] Also, PLTP activity has been linked to inflammation, microcalcification, and increased expression of osteopontin, which are histologic markers associated with plaque vulnerability and ulceration.[14]

Paraoxonase 1 (PON1) has been shown to be protective against atherosclerosis by preventing accumulation of low-density lipoprotein and cholesterol by slowing down oxidation and preserving the antioxidant function of high-density lipoprotein.[15] We found however, this protein to be enriched in our VP group. One can wonder whether this is a response to a regulatory mechanism to prevent inflammation and oxidative stress within the plaque, as is the case for lactoferrin, which poses similar antioxidant effects, and its elevation has been associated with increased cardiovascular risk.[16]

Pro-inflammatory proteins. CAMP was enriched in greater than two-fold concentration in our VP group. This peptide has been studied in coronary artery plaques, and it has been demonstrated that Cathelicidin peptide LL-37 (a subtype of CAMP) is found in reactive T-cells suggesting an immunological response and inflammatory contribution of CAMP.[17,18]

Proteinase 3 (PRTN3) is a neutrophil serine protease that has been implicated in the pathogenesis of atherosclerosis and cardiovascular disease in animal and human studies with coronary artery disease.[19,20] This protein can serve as a predictor of death or heart failure in patients with acute myocardial infarction.[19] The mechanism is related to pro-apoptotic and inflammatory properties by increasing pro-inflammatory cytokines and angiotensin.[19] Neutrophil elastase is suggested as a component in the development and progression of atherosclerosis by contributing to endothelial dysfunction, leukocyte recruitment, foam cell formation, and plaque destabilization.[21] Further, other studies suggest that the neutrophil elastase and PRTN3 might play a combined role in atherosclerotic disease progression.[20]

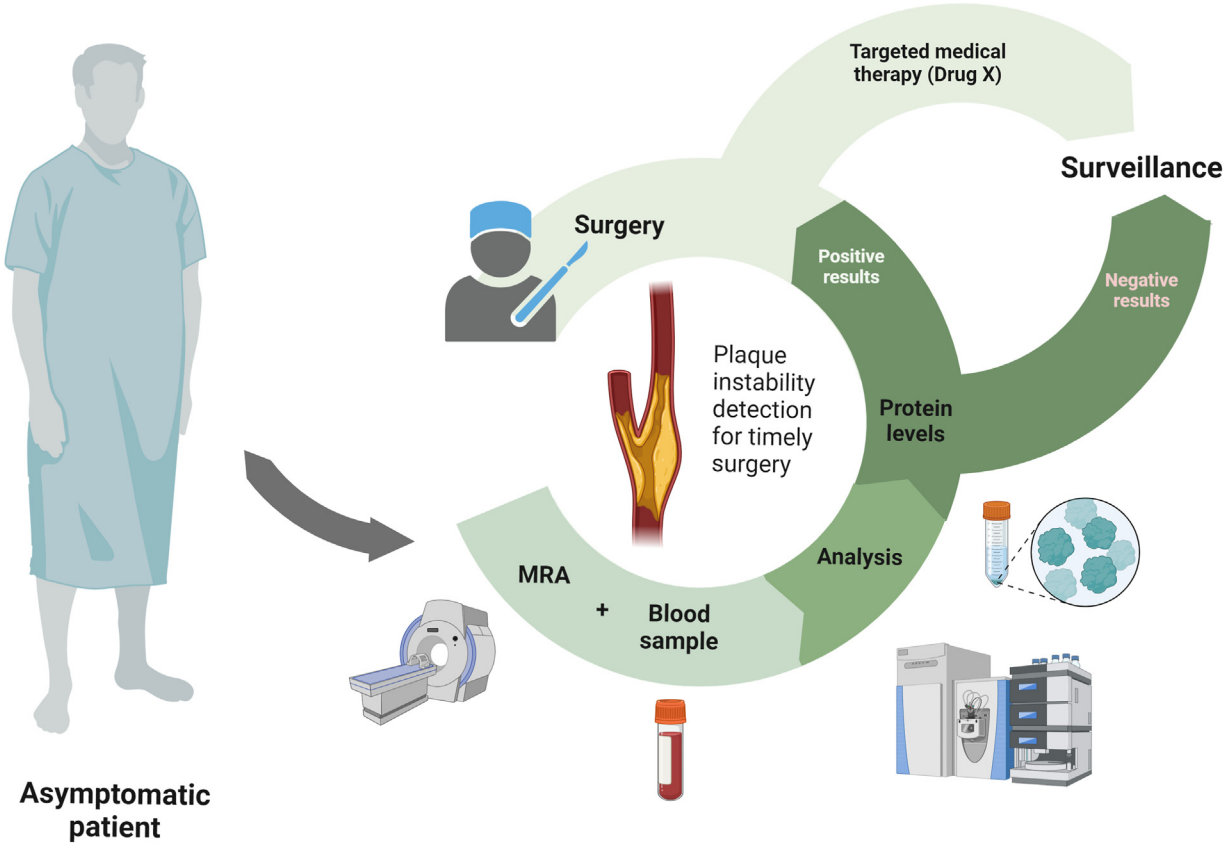

Fig 4. Proposed utility of protein identification in the clinical setting in patients with carotid atherosclerotic disease: early identification of plaque instability with timely offer for interventions to reduce risk of neurologic events. *MRA*, Magnetic resonance angiography.

Granulin (GRN) peptides have been associated with inflammatory pathways. Advanced atherosclerotic plaques demonstrate higher levels of GRN, suggesting that it may play a more prominent role in later stages of carotid atherosclerotic disease[22] by the interleukin-8 pathway.[23] Along the same lines, we found increased expression of ferritin light chain in our ulcerated plaques, which is a known biomarker of inflammation and upregulated by the presence of inflammatory cytokines.[12,24]

Other proteins associated to increased inflammation are beta 2 glycoprotein 1 (β2GPI),[25] Peptidoglycan Recognition Protein 2,[26] Phospholipase D1,[27] and adducin 3 (ADD3) polymorphisms,[28] but the underlying mechanisms remain unknown.

Proteins related to plaque progression. S100A8/12 is a calcium-binding protein and was found to be highly abundant in our VP cohort. It might play a crucial role in plaque instability. Some animal and human studies have addressed this peptide as part of the pathophysiology of carotid plaque rupture. S100A4 has proven to have a positive correlation with the degree of expansive remodeling of atherosclerotic plaques derived from smooth muscle and foam cells.[29] S100A12 is present in higher concentration in patients with carotid plaques compared with healthy subjects, and the mRNA levels of S100A8/9/12 are higher in patients with more recent onset of symptoms.[30] This protein is also actively released in unstable plaques and has a crucial role in the upregulation of the inflammatory response predisposing to plaque rupture.[31]

Another protein highly abundant is Plasma Kallikrein (Kallikrein B1), which has been found in endothelial, inflammatory, foamy macrophages, and smooth muscle cells within atherosclerotic plaques.[32] Elevated levels of circulating plasma kallikrein-kinin system components included kinin B1 receptor (KB1R), which has been associated with the presence and severity of coronary artery disease.[33] The complete list of proteins is presented in the Table. There are, however, several proteins that do not have a known role in atherosclerosis.

LIMITATIONS

Although this retrospective study offers valuable insights in patients in two distinct groups (VP vs AP), relevant limitations should be acknowledged. The study's sample size may limit the generalizability of findings potentially affecting the proteins characterized in each group. Moreover, the retrospective nature of data collection may introduce inherent biases and inaccuracies, as it

relies on medical records that may vary in completeness and quality. Furthermore, these were all patients who underwent CEA, so inherently there was risk of stroke indicating the need for the procedure. Additionally, the methods employed for sample collection and analysis may not have been standardized across all cases, introducing variability that could impact the reliability of results. Despite these limitations, these results demonstrate protein signals that could be useful for early detection of plaque instability rather than waiting for a devastating neurologic event. There are data utilizing proteomic analysis for the development of biomarkers. Our study focused on the analysis of plaque samples, and we are gearing our studies towards the potential identification of serum biomarkers found once plaque proteins for disease progression have been identified, which will further enhance our current practice by offering timely surgical interventions and/or developing targeted medical therapy associated with unique proteomic profiles. We propose Fig 4 as the future assessment of our at-risk patient population for prompt intervention and/or potential pharmacologic intervention and continued surveillance for those patients with atherosclerotic carotid artery disease by including preoperative plaque morphology assessment and serum analysis.

CONCLUSION

This study contributes to the understanding of protein biomarkers associated with carotid plaque vulnerability, offering insights into disease progression and stroke prevention. We present a comprehensive study of multiple proteins with significant bioavailability in vulnerable plaque specimens from patients after carotid endarterectomy, offering new insights regarding their role in the natural history of the disease. Further validation studies and multi-center trials will be needed to confirm the value of these potential biomarkers.

The authors acknowledge assistance from the Mayo Clinic Proteomics Core, which is a shared resource of the Mayo Clinic Cancer Center (NCI P 30 CA15083). The authors also acknowledge the internal funding sources provided by the Mayo Clinic Research Accelerator for Clinicians Engaged in Research (RACER) and RACER Plus; especially the support given by the Dean of Research, Dr Alfredo Quinones-Hinojosa, to Clinicians Advancing the Research shield at the Mayo Clinic in Florida. The authors also acknowledge the collaboration of Drs Leonard Petrucelli and Mercedes Prudencio's laboratory for their continue support at enhancing and improving the Neurosciences at the Mayo Clinic in Florida.

AUTHOR CONTRIBUTIONS

Conception and design: BM, CP-S, GP, TG, SS, YE
Analysis and interpretation: BM, CP-S, GP, KM, MC, MP, LP, JM, AP, YE
Data collection: KM, CM, MP, AN, YE
Writing the article: BM, CP-S, YE
Critical revision of the article: BM, GP, KM, MC, MP, TG, SS, AN, LP, JM, AP, YE
Final approval of the article: BM, CP-S, GP, KM, MC, MP, TG, SS, AN, LP, JM, AP, YE
Statistical analysis: Not applicable
Obtained funding: Not applicable
Overall responsibility: YE

FUNDING

None.

DISCLOSURES

None.

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Submitted May 10, 2024; accepted Jan 12, 2025.