# Recent advances in proteomic analysis to study carotid artery plaques

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## ABSTRACT

**Objective:** We sought to identify differentially expressed proteins in serum, plasma, and plaque samples of patients with carotid atherosclerotic lesions.

**Methods:** We performed a systematic review of the proteomic profile of serum, plasma, and plaque samples of patients with carotid artery disease. We included full-length peer-reviewed studies of adult humans and reported them using PRISMA guidelines. The quality of the design and content of the articles included in the review was assessed using the Newcastle-Ottawa scale.

**Results:** We included six peer-reviewed articles reporting protein expression in serum, plasma, or plaque samples from patients with carotid atherosclerosis. Three were single-center cross-sectional studies, two were single-center case-control studies, and one was a single-center cohort study. Thirty-six proteins were found to be expressed differentially when comparing samples from healthy subjects and individuals with diseased carotid vessels and between patients with symptomatic and asymptomatic carotid artery atherosclerotic lesions. Some of these were shown to be related to inflammatory or anti-inflammatory pathways in atherogenesis. CD5L and S100A12 were both found to be upregulated in patients with unstable plaque, the former owing to its anti-inflammatory properties and the latter for its pro-oxidant effects in atherosclerosis. ACTB is involved in cellular structure and integrity and was found to be downregulated in patients with ruptured carotid plaques.

**Conclusions:** Atherosclerotic carotid disease places the patient at increased risk of ischemic neurological events. Proteomics may help to understand their pathophysiological processes and can identify differential protein expression in blood samples from healthy subjects and patients with carotid artery plaques. This patient-centered approach will allow for the timely identification of individuals at higher risk of experiencing stroke. (JVS—Vascular Science 2024;5:100215.)

**Keywords:** Proteomics; Carotid artery plaque; Serum; Plasma

Carotid artery disease accounts for 15% of all neurovascular events.[1,2] For carotid artery disease, the reported stroke rate for asymptomatic patients with severe stenosis (70%-99%) is 4.7% at 5 years, with an increased hazard ratio of 2.81 in patients with a previous nonipsilateral stroke.[3] For asymptomatic patients with moderate stenosis (50%-69%), the stroke rate is 2.7% at 4 years.[4] It is hypothesized that biological and molecular changes in the plaque itself render it unstable, thereby increasing the burden for recurrence after the initial event because these plaques can rupture subsequently and embolize.[5]

Plaque characteristics such as the presence of a large lipid core, a thin fibrous cap, a paucity of smooth muscle cells and collagen, elevated inflammatory markers, and intraplaque hemorrhage have been described in at-risk plaques.[6,7] Proteomic studies may identify potential plaque and serum biomarkers of plaque instability and, therefore, aid in the management of asymptomatic patients prior to the development of a neurological event. In this systematic review, we evaluated the current literature on proteomics of plaque and serum/plasma of patients with carotid artery disease. Upregulated and/or downregulated proteins were selected and analyzed.

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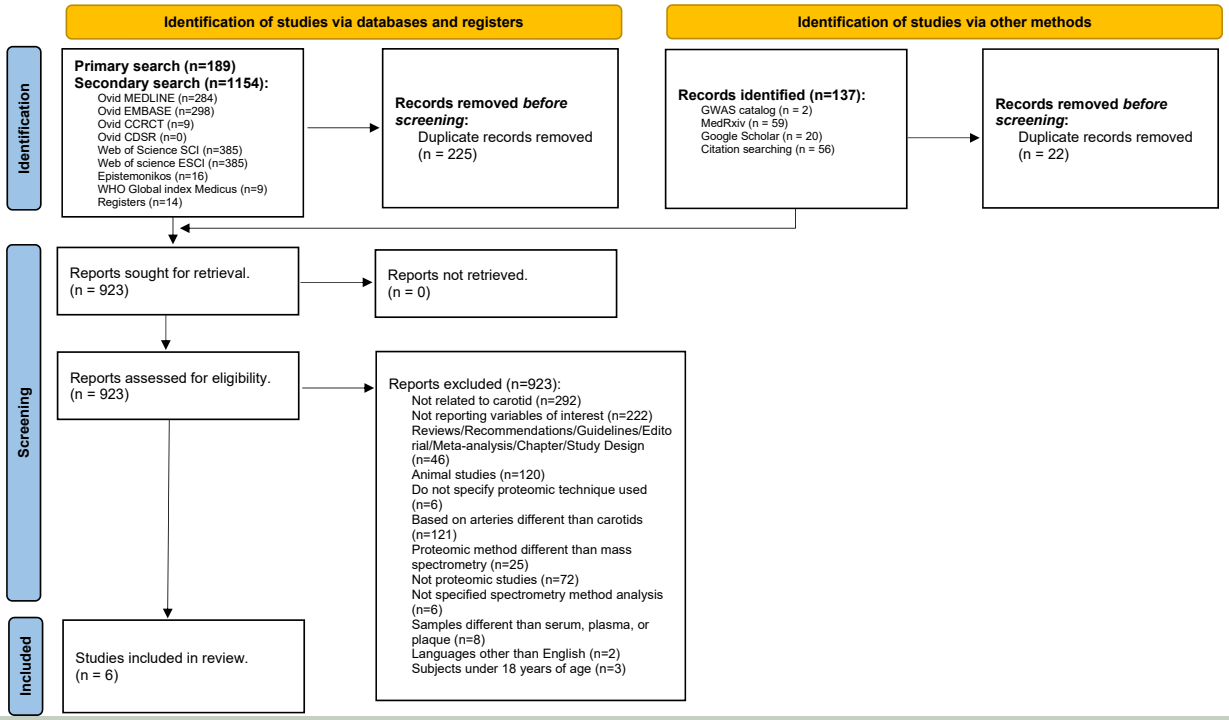


**Fig 1.** PRISMA flowchart of the systematic review.

## METHODS

**Literature search and study selection.** We conducted a thorough literature search to find all relevant literature on proteomic analyses of serum/plasma or tissue samples of patients with carotid atherosclerotic plaques. This review is being reported using the PRISMA guidelines (Fig 1). The protocol can be found on the PROSPERO International Prospective Register of Systematic Reviews (registration number: CRD42023444222).

The secondary database search strategy was developed by a health science librarian (T.B.) in consultation with the project lead (Y.E.). Studies were identified by the librarian through running searches in the MEDLINE (1946-present), Embase (1974-present), Cochrane Central Register of Controlled Trials (1991-present), and Cochrane Database of Systematic Reviews (2005-present) [all via the Ovid interface], Science Citation Index Expanded (1975-present), and Emerging Sources Citation Index (2018-present) [via the Clarivate Analytics Web of Science interface]. Gray literature resources were also searched. There were no limits to language or publication date. Filters to remove animal studies and conference abstracts were included. The search strategy was written for Ovid Medline and translated using each database's syntax, controlled vocabulary, and search fields. MeSH terms, EMTREE terms, and text words were used to search concepts of proteomics, protein-omics, proteogenomics, carotid arteries, carotid artery diseases, carotid atherosclerosis, and their synonyms. Search strategies were translated in part with the assistance of the Institute for Evidence-Based Healthcare Polyglot Search Translator.[8] All databases, registers, and grey literature resources were searched on July 11, 2023. The full search strategies are available here: https://osf.io/x5cft/.

Titles and abstracts were screened for relevance by one of the reviewers (G.C.G.). A consensus was reached with the second reviewer (Y.E.). The relevant articles were evaluated in full text to establish their eligibility using the following inclusion criteria: (a) proteomic analysis of serum or plasma samples of patients with carotid artery plaque and (b) quantitative and qualitative reports of reported results. Articles were excluded if they (a) included children, (b) were animal studies, (c) did not report quantitative results of their proteomic analysis, (d) did not specify a focus on the carotid artery, and (e) were abstracts, posters, opinions, reviews, meta-analyses, letters to editors, and editorials. The study characteristics of the articles included are presented in Table I. All database records were downloaded to EndNote 20 (Clarivate, Philadelphia, PA). Any duplicate citations were identified and removed using the methods outlined in the 2016 Bramer et al article.[13]

**Data extraction and quality assessment.** The data extracted included study details (eg, authors, date and journal of publication, study design) and patient characteristics included number of patients analyzed, sex, age, comorbidities, and medications. To assess the quality of

**Table I.** Detailed information of studies included with upregulated and downregulated proteins

| First author | Year | Samples analyzed | Study design | Patients' characteristics | Total subjects | No. of proteins measured | Proteomic analysis methods | Statistical method | Significantly upregulated proteins | Significantly downregulated proteins |
|---|---|---|---|---|---|---|---|---|---|---|
| Bao M.[9] | 2021 | Plaque (ulcerated and nonulcerated samples) | Cross-sectional | Patients who have undergone CEA | Stable plaque (n = 5) unstable plaque (n = 5) | 3082 | LC-MS/MS and HPLC-MS/MS | FDR | CD5L, and S100A12 | CKB, CEMIP, and SH3GLB1 |
| Fernandez D.[7] | 2019 | Plasma and plaque (no plaque morphology characterization) | Cohort | Symptomatic and asymptomatic patients | 46 | Not reported | CyTOF | FDR | PD-1 and IL-1 | Not reported |
| Bhosale S.[10] | 2018 | Serum | Case control | Asymptomatic and plaque-free patients | Cases: 43 Controls: 43 | 296 | MS and SRM-MS | Lasso penalized logistic regression/ ROTS | APOC3, and APOE | FBLN1C, CNDP1, CDH13, GSN, and MMP2 |
| Heo S.H.[11] | 2018 | Serum and plaque (ruptured and nonruptured samples) | Cross-sectional | Symptomatic and asymptomatic patients | 79 | 34 | MALDI-TOF MS | T-student + 2D proteomics analysis | ABCA1, APOA1, CD44, KLF2, PLIN2, and Ferritin | ACTB and α-ENO1 |
| DeGraba T.[6] | 2011 | Serum | Case-control | Symptomatic and asymptomatic patients compared with healthy controls | Cases: 38 Controls: 40 | Not reported | TOF-MS, LC-MS/MS, and MALDI-MS/MS | Random Forest model | LR9G | APOA1, VTDB, HPT, and A1AT |
| Lepedda A.[12] | 2008 | Plaque (stable and unstable samples) | Cross-sectional | Patients with severe stenosis (>70%) | 48 | 33 | MALDI-TOF MS | T-student + 2D proteomics analysis | Ferritin, SOD2, and Fibrinogen fragment D | GST, SOD3, HSP20, HSP27, Rho GD1, and annexin A10 |

*A1AT,* $\alpha_1$-Antitrypsin; *ABCA1,* ATP-binding cassette transporter A1; *ACTB,* actin beta; *ANX10,* annexin A10; *APOA1,* apolipoprotein A1; *APOC3,* apolipoprotein C-III; *APOE,* apolipoprotein E; *CD44,* cluster of differentiation 44; *CNDP1,* beta-ala-his-despeptidase; *CDH13,* cadherin-13; *α-ENO1,* alpha-enolase1; *FBLN1C,* fibulin 1 proteoform C; *GSN,* gelsolin; *GST,* glutathione S-transferase; *HPT,* haptoglobin; *HSP 27/20,* heat shock protein 27/20; *IL-1β,* interleukin-1β; *KLF2,* Kruppel like factor 2; *LRG,* leucine-rich alpha-2 glycoprotein; *MS,* mass spectrometry; *PD-1,* programmed cell death protein; *PLIN2,* perilipin2; *Rho GD1,* Rho GDP-dissociation inhibitor 1; *SOD2,* superoxide dismutase 2; *SOD3,* superoxide dismutase 3; *SRM-MS,* selected reaction monitoring mass spectrometry; *VTDB,* vitamin D binding protein.

nonrandomized studies in the review, we used the Newcastle-Ottawa scale.[14] A star-based system to evaluate each manuscript for their quality and risk of bias was performed with the following criteria: selection of study groups, the comparability of groups, and the ascertainment of exposure/outcome of interest for case-control or cohort studies (Supplementary Table I, online only). The reviewer (G.C.G.) assessed the quality of the studies, and consensus was met with the second reviewer (Y.E.).

**Outcomes assessed.** The primary outcome was the differential protein expression found in serum, plasma, and plaque of patients with carotid artery disease. The goal was to identify potential proteins that can serve as biomarkers related to plaque vulnerability. Secondary outcomes included a functional review of the proteins found to be up or downregulated in specific populations of carotid atherosclerotic disease (ie, healthy and diseased carotid arteries, symptomatic and asymptomatic patients). All studies included techniques pertaining to the proteomic profiling of plasma or serum samples, including the use of two-dimensional (2D) electrophoresis and 2D difference gel electrophoresis (2D DIGE), 2D polyacrylamide gel electrophoresis (2D PAGE), proximity extension assays, various mass spectrometry (MS) techniques including liquid chromatography with tandem MS (LC-MS/MS), mass cytometry, MS/MS, selected reaction monitoring MS, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS), TOF-MS, combined with targeted MS-based validation assays, surface-enhanced laser desorption/ionization (SELDI) chip surfaces. Validation studies were also performed with enzyme-linked immunoassays (ELISA), western blots, immunocytochemistry, and immunohistochemistry.[6-12]

## RESULTS

**Study characteristics.** Of the 13 citations, 6 articles met the inclusion criteria. Of these, three were single-center cross-sectional trials, two were single-center case-control trials, and one was a single-center cross-sectional study. Fig 1 presents a PRISMA flow diagram of the screening process and manuscript selection, and Table I summarizes the main findings of the six studies

included. The included studies were conducted from 2008 to 2021.[6,7,9-12]

All specimens were obtained from serum/plasma and/or carotid artery plaque. Bao et al[9] and Lepedda et al[12] specified the use of carotid artery plaque. Studies from Bhosale et al[10] and DeGraba et al[6] specified the use of serum; last, Fernandez et al[7] and Heo et al[11] used a combination of plaque and serum or plasma. All six studies were considered high-quality per Newcastle-Ottawa scale (Supplementary Table I, online only). Patients' characteristics can be found summarized in Supplementary Table II (online only).

The proteomic and statistical analysis techniques used for each study are presented in Table I. Bao et al[9] used LC-MS/MS, HPLC-MS/MS combined with whole transcriptome sequencing (RNA-seq) and polymerase chain reaction (PCR) while using quantitative PCR as a verification method. Fernadez et al integrated mass cytometry, cellular indexing of transcriptomes and epitopes by sequencing and single-cell RNA-seq.[7] MS/MS and selected reaction monitoring MS were applied by Bhosale et al,[10] and the latter was used as a verification method. Heo et al[11] used MALDI-TOF MS, quantitative PCR, 2D PAGE, immunohistochemistry, and ELISA as verification methods. DeGraba et al[6] used TOF-MS alongside LC-MS/MS, MALDI-MS/MS, SELDI chip surfaces, and 2D-DIGE. Last, Lepedda et al[12] used MALDI-TOF MS, 2D PAGE, immunocytochemistry, and western blot analysis.

**Proteomic analysis.** Bao et al[9] sought to identify the differences between the transcriptomic and proteomic profiles between the stable and unstable atherosclerotic plaques. By using RNA-seq analysis, they found 202 messenger RNAs (mRNAs), 488l noncoding RNAs (ncRNA)s, and 91 circular RNAs (circRNAs) that were differentially expressed; when using the HPLC-MS/MS, a total of 3082 proteins were identified, and out of these, 148 were upregulated and 145 were downregulated. To verify and validate these results, the authors compared their findings with the GSE41571 dataset from the GEO database. When compared, 30 genes were expressed differentially in both dataset GES41572 and their RNA-seq data, along with 42 genes that overlapped with their differentially expressed proteins. To identify the key genes playing critical roles in the stability of atherosclerotic plaques, differentially expressed proteins were compared to the differentially expressed mRNA, the long ncRNA (lncRNA)-target genes, and circRNA-originated genes. Only two differentially expressed proteins, cluster of differentiation (CD) CD5L and S100A12, overlapped with mRNA. Two other differentially expressed proteins, CKB and CEMIP, overlapped with the lncRNA-targeted genes, and one protein, SH3GLB1, overlapped with a circRNA-originated gene. After performing a network analysis, they found that CKB was a target gene of lncRNA MSTRG.11,344.17, CEMIP was a target gene of lncRNA MSTRG.12,845, and SH3GLB1 originated from circRNA hsacirc_000,411. The peptide false discovery rate (FDR) was ≤0.01, and the protein FDR was ≤0.01.

Fernandez et al[7] mapped the immune microenvironment of atherosclerotic plaques, identifying mirroring changes in blood and pinpointing cell-specific alterations associated with cardiovascular events, such as stroke and transient ischemic attack. A higher expression of programmed cell death protein (PD-1) was found in plaque T cells. This finding was confirmed at the transcriptional level by an exhaustion gene expression signature, like that of exhausted T cells in the tumor microenvironment. PD-1 is a marker of T-cell exhaustion and is upregulated upon T-cell activation. It is required to modulate the atherogenic responses of activated T cells in the arterial wall, and its inhibition results in plaque progression. Another finding was that IL-1 signaling was upregulated in asymptomatic patients when compared to symptomatic patients. It has been previously reported that IL-1 signals innate proinflammatory functions in macrophages and T cells.

Bhosale et al[10] based their research on the evidence that the atherosclerotic process begins early in life and may remain asymptomatic for years, so they decided to study young and middle-aged adults with nonobstructive plaques to explore serum biomarkers for early stage preclinical atherosclerosis. Seven proteins ($P < .05$) previously linked with atherosclerosis were differentially abundant. These were fibulin 1 proteoform C (FBLN1C), beta-ala-his-dipeptidase, cadherin 13, gelsolin, and 72 kDa type IV collagenase, which were less abundant, whereas apolipoproteins C-III and apolipoprotein E were more abundant. After correction for testing multiple hypotheses, only the difference in FBLN1C levels was statistically significant (FDR of <0.05). After using a machine learning approach, a combination of FBLN1C, apolipoprotein E, and cadherin 13 was found to provide the best classification of the cases from controls. They verified this finding using samples without depletion and, based on the verification data, the quantitative difference of FBLN1C remained significant.

Heo et al[11] studied the differences in molecular mechanisms underlying ruptured and nonruptured carotid plaques. Among the 34 genes that were analyzed, the expression of five RNAs (ABCA1, apolipoprotein A1 [APOA1], CD44, KLF2, and PLIN2) were significantly higher in ruptured plaques compared to nonruptured plaques. After a 2D proteomic analysis was performed, three proteins showed a greater expression between the two groups. These were ferritin, ACTB, and alpha-enolase1 (α-ENO1). Ferritin expression was significantly higher in ruptured plaques than in nonruptured plaques, while ACTB and α-ENO1 had higher expressions in nonruptured plaques. These observed differences suggest that molecular mechanisms underlying carotid plaque rupture led to increased expression of ABCA1, APOA1,

CD44, KLF2, PLIN2, and ferritin and decreased expression of ACTB and α-ENO1. ELISA was performed to detect ABCA1 in the serum obtained from both groups. ABCA1 was only detected in the serum of patients with ruptured plaques. Among those with symptomatic ruptured plaques, seven patients showed positive ABCA1 expression, whereas samples with nonruptured plaques and asymptomatic rupture had no ABCA1 expression. This protein favors atherogenesis; low serum levels of ABCA1 can lead to increased total cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-C) concentration, and decreased high-density lipoprotein cholesterol concentrations.

DeGraba et al[6] compared serum biomarkers in patients undergoing carotid endarterectomy with healthy matched controls. Of the 38 cases who underwent carotid endarterectomy, 16 were symptomatic; the remaining 22 were asymptomatic. Univariate analysis of the SELDI data comparing atherosclerosis patients to controls revealed 42 peaks with a $P$ value of$<.01$ of mean intensity. Then, to better classify the groups based on SELDI protein peak data, a random Forest analysis was applied to the dataset to obtain an unbiased estimate of correction prediction rates. Based on the significance weighing of specific peaks in the random Forest models, the four unique SELDI peaks showed distinguished atherosclerosis samples from controls. These SELDI peaks were 4.2 kDA, 16.7 kDA, 4.4 kDA, and 28 kDA. The 28.1k-DA peak was identified as APOA1. Two-dimensional DIGE analysis of the albumin-depleted serum on the 20 randomly selected samples revealed 11 spots ($P < .01$). Using MALDI-MS/MS and liquid chromatography MS/MS, 8 of the 11 proteins were identified. Four of these spots belonged to the same parental protein molecule haptoglobin, and two were related to α1-antitrypsin (A1AT). These were both downregulated in the symptomatic endarterectomy patients. Vitamin D binding protein was downregulated in the symptomatic endarterectomy, and leucine-rich-α2-glycoprotein precursor (LRG) was upregulated in the symptomatic endarterectomy group.

The Lepedda et al[12] study sought to determine if the stable and unstable human atherosclerotic plaques by histology and immunocytochemical criteria expressed different proteins. Image analysis permitted them to establish that approximately 70% of extracted proteins were of plasma origin. The analysis of 2D patterns revealed nine proteins with expression levels significantly different between the two types of plaque. When compared with the stable plaque, unstable plaque showed increased expression of ferritin light subunit (UN/ST = 2.98), superoxide dismutase 2 (SOD2) (UN/ST = 1.71), and fibrinogen fragment D (UN/ST = 1.81), and reduced expression of superoxide dismutase 3 (SOD3) (UN/ST = 0.22), glutathione S-transferase (UN/ST = 0.41), annexin A10 (UN/ST = 0.47), heat shock protein (HSP)20 (UN/ST = 0.33), and HSP27 (UN/ST = 0.44).[12]

## DISCUSSION

Understanding the pathophysiology of human atherosclerosis is a challenging task since many cellular processes are involved.[15] We summarized in Table I the most relevant upregulated and downregulated proteins in each of the articles published to date that analyze proteomics in atherosclerotic carotid artery disease. Table II offers an overview of the protein differential regarding the studied groups, and Fig 2 provides the potential implications of the protein differential in the pathogenesis of carotid artery atherosclerosis.

**Inflammatory dysregulation.** Various proteins have been found to be associated with increased inflammatory response and oxidative damage of the atherosclerotic plaque with potential roles in atherosclerosis progression and plaque ulceration. Initially, two CD proteins were shown to be upregulated.[9,11] CD5L was found to be upregulated in unstable plaques. It encodes for glycoprotein antigen protein CD5, which is involved in inflammatory regulation by promoting M2 macrophage polarization.[9] CD5L in atherosclerosis leads to increased intracellular lipid accumulation, LDL internalization, and decreased apoptosis of macrophages.[16] CD44 was also found to be higher in ruptured plaque. CD44 is an adhesion protein expressed on inflammatory and vascular cells, promoting an inflammatory cell-mediated response.[17] Increased CD44 expression in carotid plaques can be explained by the increased migration of inflammatory cells directly[11] and the secretion of this adhesion protein after plaque rupture. Also, the IL-1 pathway was found to signal innate proinflammatory functions in macrophages and in T cells, suggesting a role at the intersection of the innate and adaptive immune responses by sustaining the effector functions of T cells in asymptomatic plaques.[7]

**Inflammatory and oxidative-associated dysregulation.** Calcium-binding proteins are elevated in patients with carotid plaques, potentially indicating their involvement in plaque instability and inflammatory response leading to plaque rupture.[9,18,19] S100A12 was found to be upregulated in unstable plaques.[9] This gene belongs to the S100 protein family; it binds to the receptor for advanced glycation and end products and activates the downstream proinflammatory signals, such as nuclear factor κB and reactive oxygen species.[9] It is also involved in the pathogenesis of atherosclerosis through the S100A12-CD36 axis.[9] Ferritin light chain is also overexpressed in unstable plaques.[12] A pro-oxidant and/or antioxidant role for this protein in atherosclerosis has been suggested.[12] Ferritin synthesis is upregulated by some proinflammatory cytokines; therefore, ferritin light

**Table II.** Detailed information on upregulated and downregulated proteins in specific groups reported

| Samples analyzed | Significantly upregulated proteins | Significantly downregulated proteins |
|---|---|---|
| Ulcerated/ruptured plaque vs nonulcerated/ruptured plaque | Plaque: CD5L, S100A12, ABCA1, APOA1, CD44, KLF2, PLIN2, Ferritin *, SOD2 *, and Fibrinogen fragment D * | Plaque: ACTB, α-ENO1, GST *, SOD3 *, HSP20 *, HSP27 *, Rho GD1 *, and annexin A10 * |
| Symptomatic vs asymptomatic patients | Serum: LR9G<br>Plaque and serum: PD-1, and IL-1 | Serum: APOA1, VTDB, HPT, and A1AT |
| Asymptomatic vs healthy patients | Serum: APOC3, and APOE | Serum: FBLN1C, CNDP1, CDH13, GSN, and MMP2 |

*A1AT,* $\alpha_1$-Antitrypsin; *ABCA1,* ATP-binding cassette transporter A1; *ACTB,* actin beta; ANX10, annexin A10; *APOA1,* apolipoprotein A1; *APOC3,* apolipoprotein C-III; *APOE,* apolipoprotein E; *CD44,* cluster of differentiation 44; *CDH13,* cadherin-13; *CNDP1,* beta-ala-his-despeptidase; *α-ENO1,* alpha-enolase1; *FBLN1C,* fibulin 1 proteoform C; *GSN,* gelsolin; *GST,* glutathione S-transferase; *HPT,* haptoglobin; *HSP 27/20,* heat shock protein 27/20; *IL-1β,* interleukin-1β; *KLF2,* Kruppel like factor 2; *LRG,* leucine-rich alpha-2 glycoprotein; *PD-1,* programmed cell death protein; *PLIN2,* perilipin2; *Rho GD1,* Rho GDP-dissociation inhibitor 1; *SOD2,* superoxide dismutase 2; *SOD3,* superoxide dismutase 3; rw*VTDB,* vitamin D binding protein.

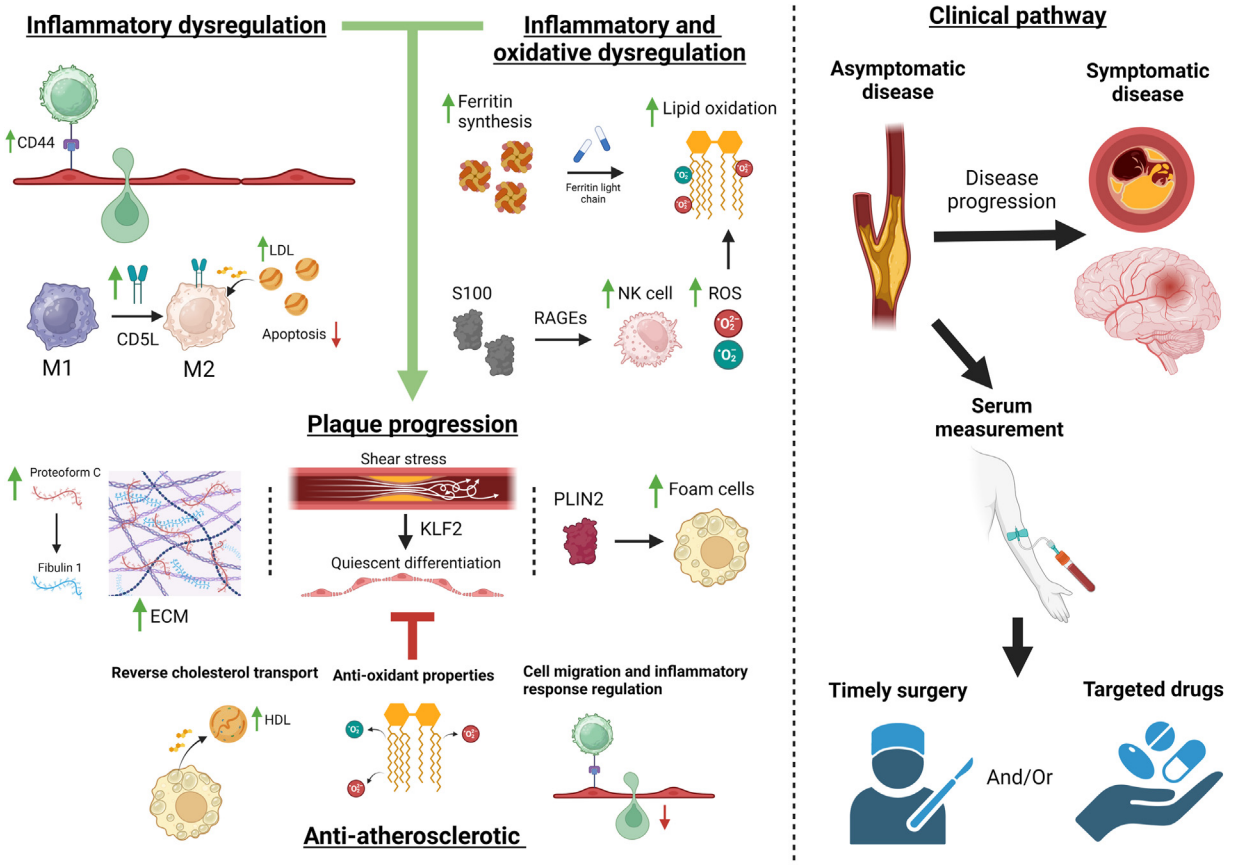


**Fig 2. (Right)** Schematic representation of proteins found in the systematic review. **(Left)** Potential clinical value of biomarkers in the setting of carotid atherosclerotic disease. Made with Biorender.com. *ECM,* extracellular matrix.

chain expression might be viewed as a marker of inflammation.[12]

**Plaque progression-related proteins.** Fibulin 1 proteoform C, an extracellular matrix protein, has shown consistently to be increased in atherosclerotic plaque formation,[10] and it has been proposed as a predictor for all-cause and cardiovascular death in patients with diabetes.[20] FLBN1 has been detected as a component of atherosclerotic lesions, and it has been suggested that decreased plasma FLBN1 could reflect its accumulation in plaque.[10] The proteoform C of fibulin 1 was downregulated in the study reported by Bhosale et al,[10] and this proteoform has been established to be the

predominant form in plasma, but it is not clear if its abundance represents a phenotype more susceptible to plaque formation or an early indication of atherosclerotic disease.[10]

KLF2 had higher expression levels in ruptured plaques. KLF2 expression is increased in endothelial cells experiencing high shear stress. Its expression establishes functional quiescent differentiation of endothelial cells; and thus, facilitates survival. An increased expression in ruptured carotid plaques can imply that their survival mechanism has been triggered owing to shear stress.[11] Also, perilipin 2 induces the formation of foam cells, aggravating atherosclerosis, which can be directly associated with carotid plaque rupture.[11]

**Antiatherosclerotic proteins.** APOA1 is protective against atherosclerosis, including reverse cholesterol transport and by promoting cholesterol efflux from foam cells. It is an early response protein and a major component of high-density lipoprotein. It also has antioxidant and anti-inflammatory properties.[11] ATP-binding cassette transporter A1 (ABCA1) expression was detected only in the serum of symptomatic patients with ruptured plaques. It has anti-inflammatory effects as a mediator of upstream proteins, NR1H2 and NR1H3, which decrease the levels of inflammatory factors, MMP9, and tissue factors in atherosclerotic environments and increase the expression of ABCA1. The immune response of ABCA1 suppresses secretion of inflammatory cytokines.[11]

Actin beta expression was lower in ruptured plaques. Actin beta is an important cytoskeletal protein associated with cell motility, structure, and integrity.[11] α-ENO1 is a target for oxidation, and its upregulation is a protective mechanism that neutralizes oxidative stress.[11] Haptoglobin is an acute-phase reactant and serves as an antioxidant by binding free hemoglobin and induces anti-inflammatory cytokines.[6] Vitamin D binding protein is a major plasma carrier of vitamin D and its metabolites. It carries out important functions, such as scavenging actin, fatty acid transport, macrophage activation, and enhancement of neutrophil and macrophage chemotaxis to C5 des Arg.[6] CKB and CEMIP, as well as their lncRNA, MSTRG.11455.17 and MSTRG.12845, were also found to be downregulated in unstable plaques. CKB encodes protein kinase B, which plays a role in the energetic hemostasis of ischemic and inflammatory disorders.[9] CEMIP encodes the cell migration-inducing and hyaluronan-binding protein, which has been reported to regulate the proliferation and migration of vascular smooth muscle cells.[9]

Glutathione S-transferase protects blood vessels against vascular toxins α,βunsaturated carbonyl, and 4-hydroxy-2-nonenale and has been implicated in the early phase of atherosclerotic lesions.[12] HSP20 and HSP27 are constitutively highly expressed in muscle cells, and they have a role in chaperone activity and in the regulation of smooth muscle tone.[12] Inhibition of Rho GDP-dissociation inhibitor 1 is a modulator of cellular responses to proinflammatory cytokines. It has been reported that, in cultured SMC, Rho dissociation inhibitor 1 is downregulated by tumor necrosis factor-α and that its expression is reduced in monocyte-derived macrophages primed with both LDL and ox-LDL.[12] The function of annexin A10 remains unknown; it is expressed predominantly in stable plaques. It can be speculated that it is involved in plaque stabilization.[12]

PD-1 is a regulator of T-cell exhaustion. It is required to modulate atherogenic responses of activated T cells and its inhibition results in aggravated atherosclerosis.[7] Some studies have addressed this protein as a key mediator of inflammation and stabilization of atherosclerotic plaques in coronary arteries.[21] In animal mice models, PD-1 stimulation led to smaller atherosclerotic lesions.[22]

**Unknown relationship to atherosclerosis.** SH3GLB1 (SH3 domain containing GRB2 like endophilin B1) was found to be downregulated. This gene encodes the endophilin-B1 or Bif-1 protein, which is involved in apoptotic and autophagic pathways. Its effects on atherosclerosis are unknown.[9] SOD3 presented a reduced expression in unstable plaque, whereas SOD2, its mitochondrial isoform, presented an increased expression in unstable plaque. The functional importance of these individual isoforms within the vessel wall, either under normal conditions or in the presence of vascular disease, is yet unknown.[12] In the vascular extracellular matrix, SOD3 represents a major defense against anion radicals, and in macrophage-rich areas of atherosclerotic lesions, it has been reported with oxidized lipoproteins and peroxynitrite-modified proteins. An increased level of SOD2 has been found in monocytes primed with lipopolysaccharide. Both SOD2 and SOD3 are regulated by inflammatory cytokines; thus, their differential expression may reflect more pronounced prooxidative and proinflammatory conditions.[12]

LRG is an acute phase reactant expressed by proinflammatory cytokines. However, LRG involvement in the pathogenesis of atherosclerosis remain to be defined.[6] A1AT forms part of a panel of acute phase reactants, which are also inflammatory-sensitive plasma proteins. It has been shown to be a major risk factor for stroke. Hypercholesterolemia, in combination with increased levels of A1AT, increases the risk of cardiovascular disease.[6] Fibrinogen fragment D increases vascular tone, endothelial disorganization and permeability to albumin.[12]

**Limitations and future insights.** Although our systematic review focused specifically on proteomic studies for potential plaque and serum biomarkers of atherosclerotic carotid disease, important limitations include

that the scope of this review was limited to carotid disease, and other studies analyzing atherosclerosis in different vascular territories may offer additional insights. Additionally, variations in study design, sample sizes, special population pathophysiological characteristics (sex-related differences, diabetic population, or other confounding factors that can alter the results), and analytical techniques might have introduced heterogeneity, which impacts the comparability and generalizability of findings. Also, most of these studies cannot ascertain whether the change in proteins preceded or followed the change in the plaque. Ancillary or advanced techniques that offer increased depth and coverage may provide improved data. Furthermore, the dynamic nature of atherosclerosis and the complexity of plaque composition may result in variability in both plaque and serum biomarker expression, potentially influencing the consistency and reliability of identified biomarkers. Finally, publication bias, with positive findings being more likely to be published than negative or null results, potentially skew the overall interpretation of the literature. This review, however, offers current knowledge on the potential role of proteomics analysis coupled with transcriptomic expression profiling in detecting different protein plaque and serum biomarkers for the prompt identification of unstable plaques and timely intervention. Future steps for clinical validation will need to assess dysregulated proteins in a Clinical Laboratory Improvement Amendments for assay development. This process involves the design, development, verification, and validation of potential proteins.

## CONCLUSIONS

Atherosclerotic carotid disease places patients at higher risk for a neurological ischemic event. Our systematic review demonstrates that proteomics can potentially identify differential protein expression in patients with carotid disease, which can be used to risk-stratify individuals with carotid plaque. This patient-centered approach will allow for a timely identification of individuals at higher risk of experiencing stroke. Larger studies are needed to standardize these results and aim to develop and validate potential serum biomarkers for risk stratification and surgical timing for stroke prevention.

## AUTHOR CONTRIBUTIONS

Conception and design: GCG, JM, BM, EO, RB, YE
Analysis and interpretation: GCG, JM, BM, MP, CPS, SS, CC, GP, LP, AP, YE
Data collection: JH, TB, YE
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