Review of serum biomarkers in carotid atherosclerosis

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ABSTRACT

Background: Carotid artery atherosclerotic stenosis is a preventable major cause of stroke, but there is still a need for definition of high-risk plaque in asymptomatic patients who might benefit from interventional therapies. Several image markers are recommended to characterize unstable plaques. The measurement of serum biomarkers is a promising method to assist in decision making, but the lack of robust evidence in the carotid environment burdens their potential as a standard of care. The goal of this review was to offer an updated state-of-the-art study of available serum biomarkers with clinical implications, with focus on those that may predict carotid symptom development.

Methods: The Cochrane Library and MEDLINE databases were searched (all until September 2018) for studies on carotid plaque and serum biomarkers of atherosclerosis. Nonhuman, basic science, and histology studies were excluded, focusing on clinical studies. Selected abstracts were screened to include the most relevant articles on atherosclerotic plaque presence, progression, instability or symptom development.

Results: Some well-established biomarkers for coronary disease are not relevant to carotid atherosclerosis and other inflammatory biomarkers, lipids, interleukins, homocysteine, and adipokines may be useful in quantifying carotid disease-related risk. Some serum biomarkers combined with image features may assist vascular specialists in selecting patients at high risk for stroke and in need of intervention.

Conclusions: Prospective studies applying a combination of biomarkers are essential to prove clinical usefulness. (J Vasc Surg 2020;71:329-41.)

Keywords: Carotid atherosclerosis; Biomarker; Stroke prevention

Carotid atherosclerosis is a major and potentially preventable cause of ischemic stroke. Carotid endarterectomy and stenting are proven techniques for primary or secondary prevention of stroke, although the number of procedures required to prevent a single stroke in asymptomatic patients remains high.[1] This situation has prompted a trend in recent literature to a nonsurgical approach, even in patients with a significant degree of stenosis.[2,3] Clinical, biochemical, and ultrasound markers, magnetic resonance (MR) plaque characteristics or transcranial cerebral doppler signals have been proposed as indicators of a high-risk plaque.[4] The recently published European Society for Vascular Surgery guidelines included recommendations that imaging features (silent brain infarction or intraplaque hemorrhage on MR imaging, stenosis progression, large plaque area, or large juxtaluminal black area on carotid ultrasound computerized plaque analysis) may be used to select patients with 60% to 99% asymptomatic carotid stenosis most likely to benefit from intervention to decrease the number of patients needed to treat.[5]

Atherosclerotic plaque contents are in a constant interaction with the circulating blood. During the process of plaque progression, specific molecules may diffuse toward the serum and hence provide information as surrogate markers of plaque presence, status, and risk of complications. A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[6] Biomarkers must satisfy several criteria: proof of concept (different levels found in patients with a certain outcome), prospective validation (the biomarker must predict development of the outcome), incremental value (added to existing markers), clinical usefulness (change of a current therapy), clinical outcomes (the use of the biomarker should improve the outcome), and cost effectiveness.[7]

Some comprehensive reviews have been published on serum biomarkers and their relation to carotid disease,[8,9] but a more clinically oriented focus should be considered to aid future decision making in asymptomatic patients.

OBJECTIVES

The goal of this review was to offer an updated state-of-the-art study of available serum biomarkers with clinical implications in asymptomatic carotid atherosclerotic stenosis patients, focusing on those that may predict

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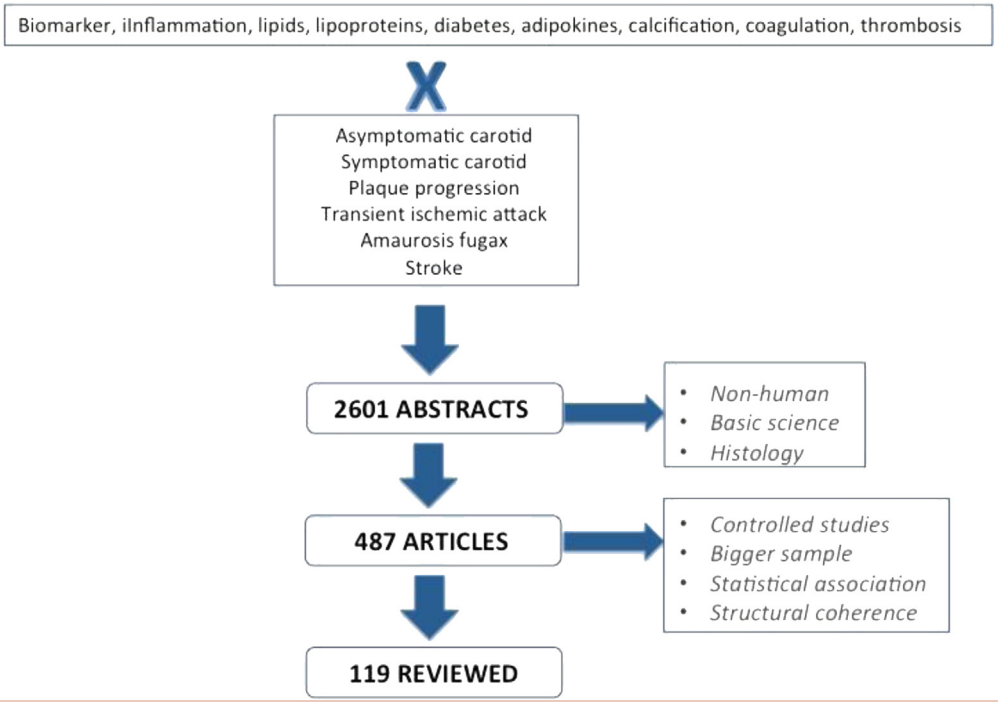

Fig 1. Search methodology. This scheme explains the inclusion and exclusion criteria for each cited study.

carotid plaque presence, instability, and symptom development to help physicians selecting patients who may benefit from surgical intervention.

METHODS

The Cochrane Library and MEDLINE databases were searched (until September 2018) for studies that evaluated associations between carotid plaque and serum biomarkers of atherosclerotic disease in humans. The terms biomarker, inflammation, lipids, lipoproteins, diabetes, adipokines, calcification, coagulation, and thrombosis were crossed-searched with asymptomatic carotid, symptomatic carotid, plaque progression, transient ischemic attack, amaurosis fugax, and stroke as keywords (Fig 1). Nonhuman, basic science, and histology studies were excluded, focusing on human studies. From 2601 abstracts on biomarker and carotid disease, 487 were selected and screened to include the most relevant (randomized controlled trials, cohort, and case-control studies over noncontrolled, larger sample size, higher statistical association, methodologic validity) and focused on atherosclerotic plaque presence, progression, instability, or symptom development. The selected articles were full-text scrutinized and are included in the References section.

Biomarker descriptions

Inflammatory biomarkers. Atherosclerosis is considered a chronic low-grade inflammatory disorder of the arterial wall (Table I). Trained immunity, a process through which innate immune cells adopt a long-term proinflammatory phenotype after brief exposure to a pathogen, such as oxidized low-density lipoprotein (ox-LDL) contributes to a persistent proinflammatory macrophage phenotype characterized by increased proatherogenic cytokine and chemokine production and increased foam cell formation at atherosclerotic plaques.[10]

High sensitivity C-reactive protein. The first described atherosclerosis biomarker, C-reactive protein (CRP), is one of the most representative acute phase proteins of the pentraxin superfamily. High sensitivity (hs)-CRP measures accurately levels of CRP to identify low but persistent levels of inflammation. According to the European Society of Cardiology guidelines for cardiovascular disease prevention in clinical practice, hs-CRP levels may be measured as part of refined risk assessment only in patients with an unusual or moderate risk profile (class IIb/B recommendation), but not in asymptomatic low-risk or high-risk individuals (class III/B recommendation),[11] whereas the American College of Cardiology/American Heart Association guidelines state that hs-CRP measurement may be considered if, after quantitative risk assessment, a risk-based treatment decision is uncertain (class IIb/B recommendation).[12] A recent large series including more than 1600 patients with asymptomatic carotid atherosclerosis prospectively followed for a median of 11.81 years, found that the risk of all-cause and cardiovascular mortality significantly increased in

Table I. Classification of biomarkers

Classification	Biomarkers
Inflammatory	hs-CRP, PTX-3, SAA, IL-6, IL-1β, TNF-α, MCP-1, suPAR
Endothelial and cell adhesion	VCAM-1, ICAM-1, L-selectin, E-selectin, endothelial MP
Matrix degrading or proteolysis	MMP-1, MMP-2, MMP-7, MMP-9, TIMP-1
Lipid	LDL, sdLDL, ox-LDL, HDL, TRL, Lp-PLA2, Lp(a), ApoA-I, ApoB, ApoE
Metabolic	Adipokines (resistin, adiponectin, FABP4), homocysteine, OPG
Hematologic	RDW, WBC count, neutrophil count, T lymphocytes, monocytes
Angiogenic and neovascularization	VEGF
Thrombosis-related	PAI-1
Other	miRNA

Apo, Apolipoprotein; *HDL,* high-density lipoprotein; *hs-CRP,* high-sensitivity C-reactive protein; *ICAM,* intercellular adhesion molecule; *IL,* interleukin; *LDL,* low-density lipoprotein; *LP(a),* lipoprotein (a); *Lp-PLA2,* lipoprotein phospholipase A2; *MCP,* monocyte chemotactic protein; *miRNA,* micro-RNA; *MMP,* matrix metalloproteinase; *MP,* microparticles; *OPG,* osteoprotegerin; *ox-LDL,* oxidized low-density lipoprotein; *PTX,* pentraxin; *RDW,* red blood cell distribution width; *SAA,* serum amyloid-A protein; *sdLDL,* small and dense LDL cholesterol particles; *suPAR,* plasma-soluble urokinase plasminogen activator receptor; *TIMP,* tissue inhibitors of metalloproteinase; *TNF,* tumor necrosis factor; *TRL,* triglyceride-rich lipoprotein; *VCAM,* vascular cell adhesion molecule; *VEGF,* vascular endothelial growth factor; *WBC,* white blood cell.

patients with elevated serum levels of hs-CRP. That risk was level response associated and patients with carotid narrowing of greater than 50% and hs-CRP levels of greater than 0.29 mg/dL had nearly twice as high a risk of cardiovascular mortality compared with patients with carotid stenosis of less than 50% and hs-CRP levels of less than 0.29 mg/dL.[13]

This association with carotid disease, however, is also controversial. Some studies suggest that high serum hs-CRP levels can predict the presence of carotid plaque,[14-16] although other studies could not establish that association[17,18] or any correlation with the degree of stenosis.[14,19] Plaque type relation to hs-CRP levels is also diverse: some studies report association with echolucent plaques,[20,21] but other studies correlated hs-CRP levels with increased plaque volume, but not with echolucency[17,22] or grey-scale median (GSM) value.[23] hs-CRP may predict plaque instability on MR imaging (hypointensity in T1 weighted images)[24] and levels 5 mg/L or greater were significantly associated with a greater number of new cerebral lesions detected on diffusion-weighted MR imaging during carotid artery stenting,[25] but did not correlate with plaque inflammation as determined by carotid artery fluorodeoxyglucose (FDG) uptake on positron emission tomography (PET).[26,27] A recently reported large series found no significant association between progression of carotid intima-media thickness (cIMT) over a 2-year period and average hs-CRP levels; values were also not related in a dose-response manner, assuming it might be considered as risk marker rather than a causal factor.[28] Nevertheless, elevated baseline hs-CRP levels were independently associated with increased ischemic stroke risk in a meta-analysis summarizing results of 12 studies, which included more than 2000 patients[29] and also predicted early restenosis after carotid endarterectomy.[30]

Pentraxin-3. Pentraxin-3 (PTX3) is another acute phase protein that has been reported associated with the presence of atherosclerotic plaques[31] and elevated levels of PTX3 were also found in patients with plaque instability undergoing carotid stenting.[24] Nevertheless, the association with the presence and severity of carotid stenosis is questioned in other studies[32,33] and a population-based study involving more than 2400 subjects, showed that PTX3 is not a predictor of incident cardiovascular events.[34]

Serum amyloid-A protein. Serum amyloid-A protein (SAA) is an acute phase apolipoprotein (Apo) related to high-density lipoprotein (HDL). Levels of greater than 10 mg/L were significantly associated with a greater number of new cerebral lesions detected on diffusion-weighted MR imaging during carotid artery stenting[25] and significantly associated with progressive atherosclerosis measured by ultrasound examination.[35] Higher levels can identify patients with ischemic stroke caused by atherothrombosis[36] vs cardioembolic stroke.

Interleukin 6. Interleukin 6 (IL-6) is a master proinflammatory and procoagulant cytokine. Related to coronary artery events, it has also been reported elevated in patients bearing carotid atherosclerotic lesions and associated with cIMT,[18] but not with the degree of stenosis.[14,15,18] IL-6 may reflect local inflammatory activity, because it is upregulated in patients with plaque instability features on MR,[24] and increased in patients who underwent carotid endarterectomy[37] and in the debris retrieved from the cerebral embolic protection devices used during carotid artery stenting.[38]

IL-1β. IL-1β is an important mediator of the inflammatory response involved in cell proliferation, differentiation, and apoptosis. Subcutaneous injection of canakinumab, a human monoclonal antibody that neutralizes IL-1β, in patients with well-controlled diabetes mellitus and

high cardiovascular risk, significantly decreased systemic inflammation (measured by levels of hsCRP and IL-6) without major effect on LDL or HDL,[39,40] supporting its significance as a mediator of atherosclerosis activity. IL-1β was also found independently and significantly associated with the presence of carotid artery stenosis in patients who underwent carotid endarterectomy.[37]

Tumor necrosis factor α. Tumor necrosis factor α (TNF-α), also known as cachectin, is a major proinflammatory cytokine involved in early inflammatory events. It is associated with a larger plaque size and has an inverse correlation with plaque GSM estimated by ultrasound examination.[23,41] TNF-α is also increased in patients with plaque instability on nonsignificantly[24] and significantly increased in symptomatic patients.[42]

Monocyte chemotactic protein 1. Monocyte chemotactic protein 1 (also known as chemokine C-C motif ligand-2) is one of the key chemokines that regulate migration and infiltration of monocytes and macrophages across vascular endothelium for routine immunological surveillance of tissues, as well as in response to inflammation. Monocyte chemotactic protein 1 was found associated with carotid artery stenosis in patients who underwent carotid endarterectomy[37] and in patients with symptomatic carotid stenosis when compared with asymptomatic carotid stenosis.[43]

Plasma-soluble urokinase plasminogen activator receptor. The soluble form of the cell-surface urokinase plasminogen activator receptor is released by endothelial and immune cells by proteolytic cleavage in an inflammatory environment. Plasma-soluble urokinase plasminogen activator receptor (suPAR) is predictive of prevalent carotid and peripheral atherosclerosis and of incident events.[44] Levels were higher in patients with symptomatic carotid stenosis[45] and among symptomatic patients, higher in those with stroke or transient ischemic attack than in those with amaurosis fugax.[46] These results may indicate that suPAR is a biomarker of the instability and severity of the thrombotic consequences of atheroma.

Endothelial biomarkers and cellular adhesion proteins. Selectins (P, E, and L) are a family of cell-surface glycoproteins involved in the rolling and anchoring of leukocytes on the vascular wall. Intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs), induce firm adhesion of inflammatory cells at the vascular surface. Expression of VCAM-1, ICAM-1, and L-selectin has been consistently observed in atherosclerotic plaques and their soluble forms have been identified in the circulation.[47]

VCAM-1. VCAM-1 levels have been reported to be positively associated with cardiovascular mortality,[48] the presence of carotid atherosclerotic lesions,[15] and MR markers of plaque instability.[24] Nevertheless VCAM-1 plasma concentration was not found to be correlated with the degree of stenosis[15] or with FDG uptake on PET imaging in the carotid artery.[26]

ICAM-1. There is evidence for a predictive role of circulating levels of ICAM-1 in initially healthy people as a significant association was observed with cardiovascular mortality.[48] ICAM-1 was found elevated in more than 300 patients who underwent carotid endarterectomy compared with healthy controls.[37]

Selectins. L-selectin is expressed on all granulocytes and monocytes and on most lymphocytes. It has been related to larger plaque size estimated by ultrasound imaging[41] in patients with carotid atherosclerotic plaque. E-selectin levels significantly associated with carotid artery stenosis in patients who underwent carotid endarterectomy.[37]

Endothelial microparticles. Endothelial microparticles (EMPs) are submicron particles (0.1-1.0 μm) released in response to endothelium cell activation or apoptosis promoting oxidative stress and vascular inflammation. EMP concentrations were significantly higher in patients with carotid stenosis (≥70%) and in asymptomatic patients with unstable plaques compared with controls.[49] Certain specific EMP subsets, were higher in unstable plaques on histologic postoperative analysis in a cohort of patients undergoing endarterectomy.[50]

Matrix-degrading or proteolysis biomarkers. Matrix metalloproteinases (MMPs) are a class of proteases involved in extracellular matrix degradation that may be involved in the process of plaque destabilization and cap erosion. An imbalance between these enzymes and their inhibitors (tissue inhibitors of metalloproteinases may lead to matrix degradation and plaque destabilization. Carotid plaques from surgery samples were analyzed histologically and features of instability were found in patients with higher serum levels of tissue inhibitor of matrix protease 1, MMP-1, and MMP-7.[51] MMP-7 was also elevated in the sera of patients who had a stroke 2 to 6 months before.[52] MMP-9 levels were higher in patients with active carotid plaques at PET imaging[27] and symptomatic patients submitted to carotid endarterectomy showed higher serum levels of MMP-2 and MMP-9.[53] Additionally, elevated levels of MMP-9 were found in the debris retrieved from the cerebral embolic protection devices used during carotid artery stenting.[38]

Lipid biomarkers. Lipid factors are, together with inflammatory factors, the main actors in the onset, evolution and destabilization of the atheroma plaque, although their role and specific functions remain to be fully understood.

LDL cholesterol. There is compelling evidence on the cardiovascular benefits of LDL cholesterol (LDL-c) lowering, mostly by decreasing the availability of cholesterol particles able to enter the endothelium at the earliest stage of atherosclerosis. However, the notion of residual cardiovascular risk recently emerged in patients treated with statins, even after achieving a significant LDL-c reduction, caused by the atherogenic effects of triglyceride-rich lipoproteins (TRLs), particularly the very low-density lipoproteins (VLDL). This finding prompted

the use of the concept of non-HDL cholesterol, which reflects cholesterol in all atherogenic particles containing Apo B (LDL-c + VLDL-c + lipoprotein(a) + cholesterol remnants). In this regard, non-HDL cholesterol might outperform LDL-c as a lipid marker of cardiovascular risk, in particular in patients with atherogenic dyslipidemia.[54] Recently, however, LDL-c, even at normal levels, was found to be independently associated with the presence and extent of early systemic atherosclerosis in the absence of major cardiovascular risk factors.[55]

LDL-c also comprises multiple distinct subfractions, which can be separated by ultracentrifugation, gradient gel electrophoresis or nuclear MR. In contrast to large LDL-c particles, small and dense LDL-c particles have a greater atherogenic potential owing to decreased clearance, greater binding, increased penetrability, and susceptibility to oxidative modification. Small and dense LDL-c particles have been associated with coronary heart disease and with carotid atherosclerosis.[56] Higher plasma levels also independently predicted an increased cIMT and associated proinflammatory activation of peripheral mononuclear cells and endothelial cells in patients with carotid atherosclerosis.[57]

Ox-LDL. Oxysterols are oxidized end products of cholesterol metabolism. Their production is enhanced by certain risks factors like tobacco exposure or overuse of frying oil, which produce oxidizing free radicals. Oxysterols are cytotoxic to cells by inducing apoptosis, stimulating the formation of foam cells, and contributing to plaque vulnerability by inducing the upregulation of MMP-9 in macrophages.[58] The ox-LDL-c plasma levels are inversely correlated with carotid plaque GSM, indicating that lipid peroxidation and reduced antioxidant capacity result in more vulnerable carotid plaques.[23,41,59]

HDL-c. HDL-c protects the vessel wall against plaque progression, inducing the transformation of plaque mass to a higher echogenicity, through the reduction of lipid content and inflammation.[60] Epidemiologic studies have proved the association of HDL-c levels and cIMT.[61] Low levels of HDL-c and higher total cholesterol/HDL-c ratios were associated with lower GSM and other characteristics of carotid plaque instability.[62,63] In a similar fashion to LDL-c, HDL-c particle sizes do matter: an HDL size of greater than 8.22 nm was independently associated with low cIMT[64] and there was an inverse association between HDL3-c (smaller molecules) and plaque area and a positive association between HDL2-c (larger and most effective in cholesterol removal) and plaque thickness.[65]

TRLs. TRLs are a pool of lipoproteins that include chylomicrons, VLDL, intermediate-density lipoproteins (IDL), and other remnant lipid metabolism particles. They can predict increased cIMT and are associated with a proinflammatory activation of peripheral mononuclear cells and endothelial cells.[57] Elevated TRL levels have been identified as an independent risk factor for future ischemic strokes and for echolucent carotid plaques.,[66,67]

Lipoprotein phospholipase A2. Lipoprotein phospholipase A2 (Lp-LPA2) travels along with circulating LDL-c and induces a proinflammatory reaction in the vessel. Its levels are higher at shoulder and necrotic lipid core areas of histology samples.[68] Secretory PLA2 levels, but not Lp-PLA2 levels, have been associated with atherosclerotic plaques and outcome.[69] Interestingly, however, circulating Lp-PLA2 was found to be increased in patients with high-grade carotid stenosis and unstable plaques in a small series of patients undergoing endarterectomy.[70]

Lipoprotein (a). Lipoprotein (a) (Lp(a)) is an LDL-like particle, rich in cholesterol and strongly influenced by genetic background. Elevated levels are associated with an increased risk for cardiovascular diseases,[71,72] but not carotid atherosclerosis in a group of patients with statin-treated familial hyperlipidemia.[73] Other studies reported increased levels of Lp(a) in patients with high-grade unstable carotid stenosis.[70] and a strong correlation with hypoechoic plaques.[74] Lp(a) apheresis resulted in cIMT and plaque reduction,[75,76] but, although Lp(a) independently predicted carotid stenosis and occlusion, it was not related with plaque area in another study.[77]

Apos. Apos are the protein components of plasma lipoproteins, which consist of a core of triglyceride and cholesterol esters and a peripheral region of phospholipid, sphingolipid and protein. The most relevant subtypes are Apo A-I (the main protein on HDL), Apo B-100 (the main protein on LDL), Apo C-II (important in chylomicrons and VLDL, activates lipoprotein lipase), and Apo E (present in chylomicrons, VLDL, and IDL, allowing the binding of these lipoproteins to the hepatocytes).

ApoA-I (or *ApoA1*) is considered an atheroprotective biomarker for its relation with HDL-c. Anti-ApoA-I immunoglobulin G levels were independently associated with cardiovascular disease in the general population and also related to cardiovascular biomarkers in secondary prevention in a recent cross-sectional population study with more than 6000 patients.[78] A meta-analysis including eight cohort and four case-control studies concluded that reduced ApoA-I, increased ApoB levels, and the ApoB/A-I ratio were risk factors for a first ischemic but not hemorrhagic stroke.[79] This conclusion is supported by a clinical study where ApoA-I levels were lower in ischemic stroke cases vs controls.[80]

ApoB, which constitutes the gross majority of Apos found in LDL, is considered atheroprone, as reported in a meta-analysis,[79] although no significant correlation with major cardiovascular events was found in a large cohort study.[81] The existence of two allele proteins (ApoB100 and ApoB48), might bias these findings, and no report of specific determination of ApoB100 related to carotid atherosclerosis has been published as yet.

ApoE presence is high among TRLs such as VLDL and IDL. A large meta-analysis of 22 studies including 30,879 participants demonstrated a significant association between APOE genotype and cIMT[82] and a positive dose-response association with LDL-c, cIMT, and ischemic stroke was proven in another large meta-analysis.[83] In contrast, no evidence of an association of circulating ApoE concentration with cardiovascular events was found in a patient cohort including more than 9000 participants, with the authors concluding that these results may be explained by isoform-specific functions.[84]

Metabolic biomarkers.

Adipokines. Adipokine is the term used to name cytokines (cell signaling proteins) secreted by adipocytes.

Resistin is involved in glucose and lipid homeostasis. As a marker of an altered metabolic status, levels were significantly higher in symptomatic than in asymptomatic subjects submitted to carotid endarterectomy.[85]

Adiponectin is also related to systemic inflammation and metabolic disease, probably by interaction with c-HDL efflux capacity (the ability of HDL to accept cholesterol from macrophages, a key step in reverse cholesterol transport).[86] Its relation with cIMT is inconclusive: some studies found a positive correlation,[87,88] although a meta-analysis including 55 studies suggested an inconclusive inverse association.[89] In a systematic review of 12 studies, circulating levels of adiponectin predicted a higher risk for ischemic stroke,[90] suggesting an instability-promoting role. Conflicting with these findings, adiponectin levels have been found positively associated with plaque GSM (indicating a more stable, lower fat content plaque).[91]

Fatty acid binding protein 4 plays an important role in the development of insulin resistance and atherosclerosis in relation to inflammation.[92] High levels have been found in patients with carotid atherosclerosis, compared with patients having suffered a noncarotid-related stroke.[93]

Homocysteine. Homocysteine is well-documented as a biomarker for cardiovascular diseases. In the carotid setting, high levels were associated with carotid plaque morphology and total carotid plaque area measured by Doppler ultrasound examination[94] and is also correlated with restenosis after carotid endarterectomy.[95] A recent study including 5393 participants older than 40 years and free of previous cardiovascular events showed that individuals with asymptomatic carotid artery stenosis had higher homocysteine levels.[96] Very interestingly, in a retrospective analysis of patients submitted to carotid endarterectomy for asymptomatic carotid stenosis, higher homocysteine levels identified a cohort for whom intensive medical therapy alone appeared to be the preferred option.[97]

Total bilirubin. Bilirubin is a potent antioxidant that has been inversely related to cardiovascular disease, cIMT,[98] carotid plaque burden,[99] and hs-CRP levels.[100] Lower levels of bilirubin correlated with silent cerebral infarction on cerebral MR, which may precede symptomatic ischemic events.[101]

Calcification markers. Osteoprotegerin (OPG) acts as a vascular calcification inhibitor. Circulating OPG levels were higher in patients bearing carotid stenosis[102] with unstable atherosclerotic plaques[103] and in symptomatic vs asymptomatic patients (who showed greater calcification).[49] Patients with echogenic plaques had lower OPG levels in a healthy population report,[104] but higher in other studies.[105]

Hematologic biomarkers. An alteration of the different subsets of blood cells may reflect a state of activation or prolonged inflammation in a nonspecific fashion. Red blood cell distribution width was found significantly associated with advanced cIMT and with significant carotid artery stenosis[106] and white blood cell count with the presence of plaque[14,17] and with cIMT measure.[107] The baseline neutrophil count was also related to a higher prevalence of echolucent plaques[108] and with the presence of microembolisms by transcranial Doppler examination.[109,110]

Immunity system cells are thought to have a role in atherosclerosis development, as certain T-lymphocyte subtypes and total monocyte count were associated with an increased cIMT.[14,18,111] Innate immunity, driven by the monocyte-macrophage complex, may also represent a factor for developing and destabilization of the atheroma plaques,[112] and a significant correlation between plaque neovascularization and circulating levels of $CD14^{+}CD16^{-}$ monocytes[113] was also reported. When all data are considered, the relationship between levels of circulating activated monocytes and carotid artery stenosis remains controversial.[114]

Angiogenic and neovascularization biomarkers. The formation of microvessels at the plaque has also been recognized as a contributing factor to destabilization and rupture. Levels of vascular endothelial growth factor (VEGF), were associated with the presence and severity of intracranial (but not extracranial) carotid stenosis[115] and inversely correlated with carotid plaque GSM,[116,117] predicting a higher presence of intraplaque neovessels as reported in histologic studies.

Thrombosis-related biomarkers. Unstable carotid artery plaques express a wide array of thrombomodulatory factors. Only plasminogen activator inhibitor-1 (PAI-1) (a fibrinolysis inhibitor) serum levels showed a negative correlation with FDG uptake in the carotid artery.[27]

Other biomarkers. Small regulatory RNA strands called micro-RNA have been related to atherosclerosis recently in both cellular and animal models. A huge variety of such molecules have been identified, and certain types may work as serum biomarkers of human atherosclerosis and carotid plaque,[118,119] but their potential in stroke prevention requires further investigation.

DISCUSSION

We have identified a vast amount of literature about biomarkers related to atherosclerosis and significant data linking biomarkers to carotid disease, but only few clinically proven associations (Table II). Atherosclerosis, as an imbalanced inflammatory reaction of the arterial wall provoked by certain stimuli, generates a wide array of circulating inflammatory molecules that can be used as biomarkers. However, any systemic inflammatory disease might increase the production of molecules like CRP, SAA, fibrinogen, or PAI-1 by the liver, thus, being nonspecific for atherosclerosis and only reflecting an inflammatory state. CRP associates inflammatory activity and cardiovascular mortality, but is only weakly related to carotid disease. This finding might limit its role as a target for pharmacologic treatment, but suggests that CRP is a call for action biomarker. In contrast, SAA and TNF-α seem to be associated with carotid plaque activity, although there is no convincing evidence of their clinical usefulness. IL levels are increasingly recognized as reliable biomarkers for cardiovascular risk. IL-6 and IL-1β seem to have a relevant role in the carotid setting, both as a marker of presence of atherosclerosis and of plaque instability. Cell adhesion biomarkers as VCAM and selectins have shown contradictory results and at present have no clinical application, whereas EMPs are specifically associated with the imbalance of endothelial function and can be useful to predict plaque instability and disease status. Metalloproteinases, as encouraging and specific as they seem to be, have limited clinical application owing to the extensive resources required for their measurement. Lipid biomarkers, together with inflammatory biomarkers, are the dominant markers in the literature. Classical molecules as LDL-c and HDL-c, together with ox-LDL, TRLs, Lp-PLA2, and Lp(a) should constitute the essential lipid profile in any patient with atherosclerosis. Apos are considered a more specific lipid biomarker, but their significance remains unclear owing to their multiple isoforms and subclasses. Adipokines (especially resistin, adiponectin, and fatty acid binding protein 4) seem to be related to different stages of plaque development and may have a cause-effect relation; the higher the levels of adipokines, the more severe the atherosclerosis is (biological gradient). There are other nonspecific metabolic markers, like homocysteine and total bilirubin, which should be considered in the clinical setting given the amount of evidence published and their wide availability. Hematologic biomarkers show diverse results and even the most targeted for atherosclerosis (activated monocytes) remain controversial and their usefulness as clinical markers for plaque vulnerability seems to be limited, especially considering the multiple potential pathways for monocyte activation. Micro-RNA strands are promising, highly specific biomarkers of unproven clinical relevance.

Most of these biomarkers show a positive association with carotid atherosclerosis (an increased biomarker level associates a more severe atherosclerosis presence or manifestations), except for HDL-c, Apo A-I, and total bilirubin, which are inversely associated, granting them, arguably, an atheroprotective role.

There are some biomarkers patient specific enough to suggest a potential role as a target for tailored medical therapy, namely EMPs, ApoE subtypes, micro-RNA, and patterns of monocyte activation, but they are not widely available and evidence is still limited.

Several studies aim to correlate image biomarkers of plaque instability (ultrasound GSM, cerebral lesions and hypointense plaque on MRI, plaque activity at PET scanning) with serum biomarkers, both as a support for imaging findings and as surrogates for imaging studies. Inflammatory (hs-CRP, PTX-3, SAA, IL-6, and TNFα), lipid (oxLDL, HDL, TRL, Lp(a), and Lp-PLA2), also EMPs, MMP-9, adiponectin, total bilirubin, neutrophil count, VEGF, and PAI-1 (Table II), have the potential to enhance the definition of plaque at risk for complication, selecting asymptomatic patients for interventional therapies.

Making a recommendation of a specific set of biomarker tests, therefore, is cumbersome. As mentioned, some clinically proven associations are nonspecific, other promising biomarkers have little clinical associations so far, and other clinically relevant biomarkers such as metalloproteinases, ILs, or micro-RNAs require great economic resources and long determination times, limiting their use to really selected patients.

If we reconsider biomarker criteria, all those included in this review have demonstrated proof of concept, some show prospective validation (SAA, TNF-α, suPAR, MMP2, MMP9, oxLDL, TLR, Lp(a), VEGF), but some are mere findings in a retrospective revision. Most of the newer biomarkers are costly (MMPs, ILs, microparticles, micro-RNA) and only few (hsCRP, IL-1β) complement other well-known biomarkers of carotid risk (eg, degree of stenosis, plaque echogenicity, silent microinfarcts) in clinical guidelines.

The lack of robust evidence may withhold any change of behavior (in diagnosis or treatment) of a physician facing a carotid atherosclerosis patient. Furthermore, specificity is the Achilles' heel of biomarkers, both for atherosclerosis (among inflammation markers) and carotid (among atherosclerosis) markers. Our work was intended to focus on a relatively small number of clinically proven biomarkers, then selecting which ones would be of a greater use by means of cost and availability, to finally suggest a biomarker set that clinicians may use. With that in mind, we suggest a determination of nonspecific biomarkers of atherosclerosis activity as hs-CRP, homocysteine, and total bilirubin to identify subsets of patients with a higher

Table II. Biomarker associations

Biomarker	Carotid IMT	Carotid plaque	Risk features	CV events	Carotid symptoms	Determination cost
hs-CRP	[a]	[a]	[a]	[b]	[b]	↑
PTX-3		[a]	[b]	[c]		±
SAA			[b]		[b]	↓
IL-6	[b]	[c]	[b]			↓
IL-1β		[b]				↓
MPO			[a]			±
TNF-α		[b]	[b]		[b]	↓
MCP-1		[b]			[b]	↓
suPAR		[b]			[b]	↓
VCAM-1	[b]		[a]	[b]		↓
ICAM-1		[b]		[b]		↓
L-Selectin		[b]				↓
E-Selectin		[b]				↓
EMPs		[b]	[b]			↓
MMP-1					[b]	↓
MMP-2					[b]	↓
MMP-7					[b]	↓
MMP-9			[b]		[b]	↓
TIMP-1					[b]	↓
sdLDL	[b]	[b]		[b]		±
Ox-LDL			[b]			±
HDL	[b]	[b]	[b]	[b]		↑
TRL	[b]	[b]	[b]		[b]	↑
Lp-PLA2		[b]	[b]		[b]	↑
Lp(a)		[a]	[b]	[b]		↑
ApoA-I				[b]	[b]	↑
ApoB				[c]		↑
ApoE	[b]			[c]	[b]	±
Resistin					[b]	↓
Adiponectin	[a]		[b]			↓
FABP4		[b]		[b]	[b]	↓
Homocysteine		[b]			[b]	↑
Total bilirubin	[b]	[b]	[b]	[b]		↑
OPG		[b]	[a]		[b]	↑
RDW	[b]	[b]				↑
WBC count	[b]	[b]				↑
Neutrophil count			[b]		[b]	↑
T lymphocytes	[b]			[b]		↑
Monocytes	[b]	[a]				↑
VEGF			[b]			↓
PAI-1			[b]			↓
miRNA		[b]				↓

↑, low cost determinations (<5.00€/$5.50); ±, medium cost (5.00-25.00€/$5.50-$28.00); ↓, high cost determinations (>25.00€/$28.00); *Apo,* Apolipoprotein; *Carotid IMT,* carotid intima-media thickness associated; *Carotid plaque,* carotid plaque presence associated; *Carotid symptoms,* association with stroke or transient ischemic attack; *CV events,* cardiovascular morbidity and mortality associated; *EMP,* endothelial microparticle; *HDL,* high-density lipoprotein; *hs-CRP,* high-sensitivity C-reactive protein; *ICAM,* intercellular adhesion molecule; *IL,* interleukin; *LP(a),* lipoprotein (a); *Lp-PLA2,* lipoprotein phospholipase A2; *MCP,* monocyte chemotactic protein; *miRNA,* micro-RNA; *MMP,* matrix metalloproteinase; *MPO,* myeloperoxidase; *OPG,* osteoprotegerin; *ox-LDL,* oxidized low-density lipoprotein; *PAI,* plasminogen activator inhibitor; *PTX,* pentraxin; *RDW,* red blood cell distribution width; *Risk features,* plaque instability image (ultrasound examination, magnetic resonance imaging) features association; *SAA,* serum amyloid-A protein; *sdLDL,* small and dense low-density lipoprotein cholesterol particles; *suPAR,* plasma-soluble urokinase plasminogen activator receptor; *TIMP,* tissue inhibitors of metalloproteinase; *TNF,* tumor necrosis factor; *TRL,* triglyceride-rich lipoprotein; *VCAM,* vascular cell adhesion molecule; *VEGF,* vascular endothelial growth factor; *WBC,* white blood cell.

[a] Association unclear.

[b] Association proven.

[c] Proven not associated.

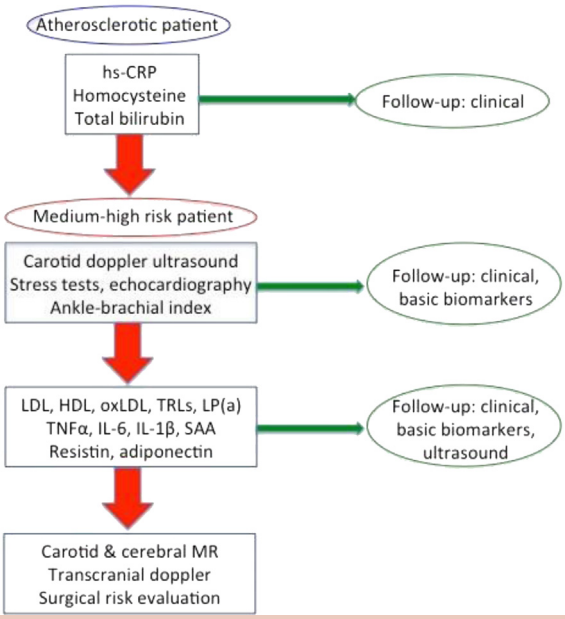

Fig 2. Patient-specific decision tree. This scheme explains the tests and follow-up that a patient should receive. *HDL,* high-density lipoprotein; *Hs-CRP,* high-sensitivity C-reactive protein; *IL,* interleukin; *LDL,* low-density lipoprotein; *LP(a),* lipoprotein (a); *MR,* magnetic resonance; *SAA,* serum amyloid-A protein; *TRL,* triglyceride-rich lipoprotein; *TNF-α,* tumor necrosis factor-α.

cardiovascular risk. Those patients should be examined more specifically, inquiring into which atherosclerosis-prone territory might be at a greater risk (coronary, carotid, limbs) with specific tests. If a significant carotid stenosis is estimated on Doppler ultrasound examination, a focused set of biomarkers including an extended lipid profile (LDL-c, HDL-c, ox-LDL, TRLs, and Lp(a)), adipokines (resistin and adiponectin), and inflammatory biomarkers (TNF-α and when possible SAA, IL-6, and IL-1β) could help the clinician in risk stratifying the patient. The addition of other proven biomarkers as the ultrasound characteristics of the plaque (hypoechogenicity, ulceration, irregularity), the presence of microemboli in transcranial Doppler and carotid plaque MR features of instability,[4] could provide the vascular specialist with support for interventional treatment in addition to medical treatment (Fig 2). The relative weight and the prognostic potential of all of these biomarkers (serum, ultrasound, imaging) is yet to be assessed, and a risk score including those biomarkers with more robust supporting evidence should be used to risk stratify patients with asymptomatic carotid disease in further prospective trials.

CONCLUSIONS

A basic biomarker profile including hsCRP, homocysteine, and total bilirubin may identify patients with evolving atherosclerosis. An extended profile including inflammatory biomarkers such as TNF-α, SAA, IL-6, and IL-1β, as well as lipid markers including LDL-c and HDL-c, ox-LDL, TRLs, Lp(a), and adipokines when available should be assessed if significant asymptomatic carotid atherosclerosis is found. Combined with imaging features, these serum biomarkers may identify asymptomatic patients at high risk for stroke. Further investigation in larger cohorts is still required to establish clinical relevance and usefulness of a specific set of biomarkers and their combination with other risks markers.

AUTHOR CONTRIBUTIONS

Conception and design: EM, VR
Analysis and interpretation: EM, JM
Data collection: EM
Writing the article: EM, JM
Critical revision of the article: EM, JM, VR
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