Review

Update on Biomarkers Associated with Large Artery Atherosclerosis Stroke

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Update on Biomarkers Associated with Large Artery Atherosclerosis Stroke

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Abstract: Intracranial and extracranial large artery atherosclerosis (LAA) are a main cause of ischemic stroke. Biomarkers may aid in the diagnosis of LAA and help to stratify patients´ risk of stroke. We performed a narrative review of the literature mainly published in the last five years with the aim of identifying biomarkers associated either with intracranial or extracranial LAA in humans. Several potential biomarkers of LAA mainly related to lipid pathways and inflammation have been studied. Diagnostic biomarkers of LAA were evaluated by measuring biomarkers levels in patients with LAA stroke and other stroke etiologies. Some biomarkers were associated with prognosis of LAA stroke as evaluated by the modified Rankin score. Increased levels of IL-6 and have been associated with the risk of progression of the atherosclerotic disease. Overall, in most studies, the results were not externally validated. External validation of these results is needed. In the future, biomarkers could be useful for the selection of patients for clinical trials. To adopt these biomarkers in clinical practice we will need robust multicentric studies proving their reproducibility and a clear practical applicability for their use.

Keywords: atherosclerosis, stroke, carotid, stenosis, biomarker, plaque, inflammation, IL-6, metalloproteinases, lipids

1. Introduction

Large artery atherosclerosis (LAA) is one of the most common causes of ischemic stroke. Its definition implies clinical and brain imaging findings of either significant stenosis or occlusion of a major brain artery (carotid or vertebral arteries) or of a branch cortical artery (anterior, middle, or posterior cerebral arteries) [1].

LAA can cause a stroke by two pathophysiological mechanisms: hypoperfusion due to a hemodynamically stenotic vessel, or by atheroembolism when there is plaque rupture or ulceration with thrombus formation and upstream embolism [2].

As the name implies, atherosclerosis is the underlying pathophysiology in LAA stroke. Atheroma is typically located at the bifurcation of arteries, where turbulent flow is highest. Several mechanisms like induced endothelial dysfunction, potentiated by inflammation and hypercholesterolemia, lead to increased permeability, with entrance of oxidated low-density lipoproteins in the subendothelial space of the intima. At the endothelial surface, the expression of adhesion molecules initiates platelet aggregation and lymphocyte/monocyte adhesion and infiltration, while at the intima layer, monocytes mature into macrophages, which take up oxidized low-density lipoprotein, and transform into foam cell. As this occurs, vascular smooth muscle cells shift from a contractile phenotype to an active synthetic phenotype, producing extracellular matrix and gradually transforming the lesion into a fibrous plaque [2]. Unstable plaques usually contain macrophages and T Lymphocytes that secrete cytokines [3]. During this process, drivers of atheroma formation that are being produced can be released and detected in the blood stream. These substances can be potential biomarkers useful to clarify stroke etiology and to stratify patients regarding their risk of stroke or progression of the plaque of atheroma.

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In this article, we aimed to perform a narrative review of the literature published in the last five years regarding biomarkers associated with LAA stroke in humans.

2. Biomarkers and their usefulness in stroke

Biomarkers are objective indicators used to assess normal or pathological processes, evaluate responses to medical interventions, and/or predict outcomes [4]. They can be biochemical measurable components, genetic information or physical characteristics of tissue picked up by imaging techniques. The ideal biomarker should be easily accessible, standardized, highly sensitivity and specific, easily interpretable, cost-effective and add value.

Biomarkers can help in stroke diagnosis, particularly in LAA stroke, for particularly two reasons:

- First, even though stenosis severity is the primarily used parameter for risk evaluation, being applied by medical guidelines to decide treatment (medical vs surgical), it is known that several high grade stenoses are asymptomatic, with a low rate of symptomatic conversion. On the contrary, there seems to exist a higher risk among low grade stenosis, with a substantial proportion of symptomatic patients and a risk of recurrent ipsilateral stroke as high as 8% at three years. [5] Biomarkers can help to understand the real risk of symptomatic conversion of these patients and act accordingly.
- Secondly, LAA biomarkers may be useful to identify stroke cause in patients classified as having an undetermined etiology. The problem persists when patients have more than one possible cause for stroke [4,6]. Having a biomarker that provides clues to a possible etiology may allow the clinician to best tailor preventive measures to the individual patient and plan their follow-up.

3. Biomarkers associated with LAA stroke

Several studies have identified different substances which can be biomarkers of LAA stroke.

3.1. Chemical biomarkers

Cholesterol particles remain one of the most recognized biomarkers of LAA stroke. Low density lipoprotein cholesterol (LDL-c) is one of the most well known biomarkers associated with LAA. It is independently associated with the presence and extent of subclinical early systematic atherosclerosis. It is currently used as a target for medical treatment. [7,8]

More recently, some studies analyzed other lipid parameters, which may be helpful to predict vascular risk.

Lipoprotein(a) Lp(a) was evaluated in a Chinese cross-sectional study that included 75305 adults with Lp(a) measurements that underwent carotid ultrasound [9]. A multiple logistic regression analysis showed that participants with Lp(a) levels ≥50 mg/dL had an increased risk of carotid intima-media thickness (cIMT)≥1.0 mm (OR = 1.138, 95% CI, 1.071-1.208) and carotid plaque (OR = 1.296, 95% CI, 1.219-1.377) compared with those with Lp(a) levels <50 mg/dL [9].

Soluble lectin-like oxidised low-density lipoprotein receptor 1 (sLOX-1) is a soluble scavenger receptor released by protease hydrolysis of LOX-1 on the cell surface, and therefore, sLOX-1 levels reflect the expression level of LOX-1 [10]. Serum soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) has been shown to be associated with the progression of atherosclerosis in endothelial cells and to be an independent predictor of functional outcome in patients with LAA ischemic stroke [11]. It also been shown to be associated with vulnerability of intracranial plaques and suggested to be useful as a supplement to high-resolution magnetic resonance vessel wall imaging to predict stroke recurrence [10]. A study by Bang *et al* analyzed the association between serum lipid indices other than LDL-c and the occurrence of symptomatic cervicocephalic atherosclerosis in a retrospective study using data from a prospective registry. This study included 1049 patients, divided between a LAA stroke group (n=247) and a non-LAA group (n=802). Patients with LAA stroke had higher triglyceride: high density lipoprotein cholesterol (HDL-c) ratio and non-HDL-c levels. After adjusting for age, risk factors, body mass index and premorbid statin use, the highest quartile of triglycerides was significantly associated with the occurrence of LAA stroke. These findings could explain why atherosclerosis in some patients progresses even after attaining intensive LDL-c reduction. Triglycerides have been reported to be a driving factor behind the progression of mild-to-moderate non-hemodynamically significant stenotic lesions [8].

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Triglyceride levels were also studied by Jiang *et al*, which looked at insulin resistance and its marker triglyceride-glucose index [12]. This retrospective cross-sectional study looked at 2836 patients admitted to the Ma'anshan People's Hospital due to acute ischemic stroke, again divided between a LAA stroke group (*n*=458) and a non-LAA stroke group (*n*=2378). Age, hypertension and the triglyceride-glucose index were identified as predictors of LAA stroke, with a positive correlation between the triglyceride-glucose index and LAA stroke incidence, even after adjusting for related risk factors. While the retrospective design of the study prevents further conclusions, the authors hypothesize that the high triglyceride-glucose index contributed to unstable atherosclerotic plaques by affecting inflammatory active substances, and subsequently led to clinical events [12].

Other parameters not directly involved in lipidic pathways have been studied as possible biomarkers of LAA stroke. Cystatin C (CysC), a marker of renal function that is a risk factor for cardiovascular disease was found to be independently associated with symptomatic extracranial internal carotid artery (ICA) stenosis, but not with intracranial ICA/middle cerebral artery stenosis in Japanese patients with noncardioembolic stroke [13].

Plasma levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) were reported to be significantly lower for LAA patients than controls (P<0.001) [14]. In a Chinese study that included 132 LAA stroke patients and 60 control patients, plasma TRAIL level was negatively correlated with prognosis evaluated with the modified Rankin scale at 3 months (r = - 0.372, P <0.001). The optimal cut-off value of TRAIL for prognosis was 848.63 pg/mL. The sensitivity and specificity at this cut-off value were 63.1% and 86.2%, respectively [14]

Levels of adiponectin and endothelial progenitor cells (EPCs) in LAA stroke patients (n=127) were found to be significantly lower compared with matched controls (n=58) ($p < 0.05$) [15]. Both adiponectin and EPCs have been proposed to have anti-atherosclerosis effects [15].

3.2. Inflammatory biomarkers

Inflammation is involved in the development, progression, and rupture of atherosclerotic plaques. Several inflammatory substances have been studied as possible biomarkers of atherosclerosis and LAA stroke. C-reactive protein (CRP) and, in particular, high-sensitivity CRP (hs-CRP) has been associated with the presence of unstable carotid artery stenosis. TNF-α, an inflammatory cytokine, numerous cell adhesion molecules and also matrix metalloproteinases were also shown to have a positive correlation. The rationale for each one is easily understandable when considering the pathophysiological process of atherosclerosis [7].

More recent studies continue to attest the relation between LAA stroke and inflammation. A study by Wei & Quan, which consisted in a protein-protein interaction (PPI) study done with resource to big data tools, found that the pertinent nodes of the PPI network for LAA included Nuclear Factor Kappa B Subunit 1 (NFκB1), interleukin-6 (IL-6), TNF- α and apolipoprotein B, which are right at the center of the inflammatory atherosclerotic cascade [16].

In a study, that included 75 Spanish patients with first ever symptomatic intracranial stenosis, prospectively followed, progression of symptomatic intracranial LAA was associated with a proinflammatory state [17]. Patients with intracranianal LAA showed increased levels of CRP (CRP>5.5 mg/L; HR, 5.4 [2.3 to 12.7]; P=0.0001) and plasminogen activator inhibitor-1 (PAI-1) (PAI-1>23.1 ng/mL; HR, 2.4 [1.0 to 5.8]; P=0.05) [17].

In a Swedish study that included 162 patients with cryptogenic stroke and 73 patients and in which blood was collected in the acute phase and after 3 months, incrased eotaxin and monocyte chemoattractant protein (MCP-1) were the main markers suggestive of occult atherosclerosis [18]. Eotaxin is a selective chemoattractant for T2 lymphocytes, basophils and eosinophils that is released by activated endothelial cells.

A small study that included 58 patients with carotid stenosis showed that patients with a vulnerable plaque showed upregulation of the proinflammatory cytokines (IL-6 and TNFα), endothelial activation markers (E-selectin and vascular cell adhesion molecule 1), and inflammation markers (hs-CRP and pentraxin (PTX3)) and downregulation of the anti-inflammatory markers (adiponectin and IL-10) [19]. PTX3 levels were higher in the vulnerable plaque group than in the stable group [19]. Vulnerable plaque was defined in this study by using ultrasonography and included low-echo plaques and mixed-echo plaques [19].

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In recent years, IL-6 has been on the spotlight as a biomarker of atherosclerosis. It is a proinflammatory cytokine secreted by activated monocytes, macrophages, endothelial cells, adipocytes, fibroblasts and T-helper-2 cells, typically after stimulation by interleukin-1 or TNF- α. In a prospective population-based cohort study, that included 4334 patients, and in which Duplex carotid ultrasound was performed at baseline and at 5 years of follow-up, log IL-6 predicted plaque severity (β=0.09, P=1.3×10^{-3}), vulnerability (OR, 1.21 [95% CI, 1.05-1.40]; P=7.4×10^{-3}, E-value=1.71), and progression (OR, 1.44 [95% CI, 1.23-1.69], P=9.1×10^{-6}, E-value 2.24) [20]. In participants with a >50% predicted probability of progression, mean log IL-6 was 0.54 corresponding to 2.0 pg/mL. The authors of this work hypothesized that the 2.0 pg/mL cutoff of IL-6 could facilitate the selection of individuals that would benefit from anti-IL-6 drugs for stroke prevention [20]. A systematic review by Papadopoulos *et al,* which reunited over 2500 patients, established higher levels of IL-6 as a risk factor for incident ischemic stroke. Unfortunately, no stroke etiology analysis was presented. [21]

Serum chitinase-3-like protein 1 (YKL-40) level has also been found to be a significant and independent biomarker to predict the clinical outcome of LAA stroke [22]. YKL-40 is a glycoprotein produced by inflammatory, cancer and stem cells.

Regarding count of blood cells, a high ratio of monocytes to lymphocytes (MLR) has been reported to be a predictor of poor prognosis in patients with LAA [23]. In a retrospective study that included 588 Chinese patients with acute ischemic stroke and 309 healthy controls without carotid plaques, the admission neutrophil-to-lymphocite ratio was found to be an independent predictor of vulnerable carotid plaque after controlling for age, gender, diabetes mellitus, systolic blood pressure [24].

Also, neutrophil extracellular traps (NETs) that exhibit pro-inflammatory and pro-thrombotic properties have been studied in patients with carotid artery stenosis. In a study that included 39 consecutive Japanese patients that underwent carotid artery stenting with dual protection and in which local arterial blood was aspirated at the stent site to measure peptidylarginine deiminase 4 (PAD4), which is essential for the formation of NETs, in a multiple linear regression analyses, PAD4 was correlated with the neutrophil to lymphocyte ratio (p = 0.01) and ulceration (p = 0.01, cut-off value: 0.49 odds ratio: 19.3) [25].

3.3. Metabolomics

A study that analyzed, by non-targeted metabolomics based on liquid chromatographymass spectrometry, 49 patients with LAA and 50 patients with small artery occlusion (SAO) and 50 matched healthy controls found differences in the metabolic profiling between the LAA and SAO groups. There were eight different metabolites, including L-pipecolic acid, 1-Methylhistidine, PE, LysoPE, and LysoPC, which affected glycerophospholipid metabolism, glycosylphosphatidylinositol-anchor biosynthesis, histidine metabolism, and lysine degradation [26].

3.4. RNA biomarkers

MicroRNAs (miRNA) are small, noncoding RNA particles, composed of 18 to 22 nucleotides that participate at the post-transcriptional level of gene expression, inhibiting translation or causing degradation of the messenger RNA. Multiples studies have documented their role in multiple biological processes and diseases, namely stroke and atherosclerosis. The Tampere vascular study assessed miRNA expression profiles in human atherosclerotic plaques and compared them with nonatherosclerotic arteries. They found 10 miRNA with a statistically significant difference in expression, of which miR-21, miR-34a, miR-146b-5p and miR-210 had a higher expression in atherosclerotic arteries. The genes regulated by those miRNAs were involved in signal transduction, regulation of transcription and vesicular transport [2].

While there may be many possible miRNA biomarkers, they may also be more specific than the conventional ones. A study by Xuan *et al* assessed only miR-137, a miRNA involved in the function of vascular endothelial and smooth muscle cells and also angiogenesis, as a possible biomarker for cerebral atherosclerosis diagnosis [27]. They compared miR-137 expression between 52 patients admitted with LAA ischemic stroke and 46 controls. As expected, they found a downregulation of miR-137 expression in patients with LAA stroke, which was well correlated with other assessed biomarkers for that disease (total cholesterol, LDL-c and hs-CRP). However, when comparing different biomarkers, the area under the curve (AUC) for miR-137 was 0.908 (specificity of 87%), while

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the AUCs for total cholesterol, LDL-c and hs-CRP were 0.810 (specificity of 71.7%), 0.819 (specificity of 84.8%) and 0.624 (specificity of 32.6%), respectively. They concluded that miR-137 had a high diagnostic value for LAA stroke etiology, with a specificity higher than other established biomarkers for atherosclerosis [27].

There are some research studies on the association between the expression of small non-coding microRNAs with carotid plaque development and vulnerability [28]. However, data remains inconsistent. Also, all major studies on carotid atherosclerotic plaque were conducted on cell cultures or animal models and very few were conducted on humans. Therefore, their results cannot be automatically extrapolated to processes in humans [3,28]. There is a lack of are robust multicentric studies proving their reproducibility and a clear practical applicability for their use [2].

MiRNAs, however have features that favor their use as biomarkers such as being highly stable in blood and having a good correlation between plasmatic and tissue levels, that way being easily accessible and reliable. Also, new well-defined protocols have been developed for their detection, extraction and isolation. Finally, with the recent development of oligonucleotide/antisense therapies, miRNA remain as a good hypothetical therapeutic target. Regulating of its expression could theoretically modulate disease development and effectively prevent the atherosclerotic process.

More recently, circular RNA (circRNA), a different type of RNA molecule, has been studied as an ischemic stroke biomarker. These molecules are evolutionarily conserved and participate in regulatory functions, acting as sponges to sequester miRNA or RNA-binding proteins, that way modulating the expression of target genes. They are claimed to be more stable than other linear RNAs due to their resistance to endonuclease activity. A Spanish study profiled circRNAs in the peripheral blood of 30 patients admitted due to ischemic stroke using a circRNA array and found different profiles when comparing atherosclerotic versus cardioembolic stroke [29]. The circRNA that were identified were predicted to interact with miRNAs involved in fatty acid biosynthesis and metabolism, lysine degradation, arrhythmogenic right ventricular cardiomyopathy, adrenergic signaling in cardyomyocytes and hypertrophic cardiomyopathy, some of which are processes related to atherosclerosis. However, the underlying mechanisms remain unclear and this was also a study with a small sample size [29].

3.5. Genetic biomarkers

Genetics holds many answers to pivotal questions regarding disease processes, even though we may not have the correct tools to understand them yet. This same premise also applies to biomarkers in LAA stroke, with genetic polymorphisms being assessed as potential ways to solve clinical problems.

Considering atherosclerotic ischemic stroke, cystatin C (CysC) is known to be involved in atherosclerotic plaque remodeling, being independently associated with cerebral artery stenosis and prognosis in stroke patients. With that in mind, two selected single-nucleotide polymorphisms of the *CST3* gene were evaluated in 3833 subjects as possible biomarkers of LAA stroke. This was a multicenter, prospective registry study occurring only in the Chinese population. No statistically significant association was found between any allele and the occurrence of LAA stroke, however carriers of the T alelle of SNP rs13038505 tended to have a lower proportion of LAA stroke. The specific functions of the two SNPs and the causal relationship between CysC concentration remains to be clarified. [30]

Another study looked at methylation alteration patterns in candidate genes in LAA stroke, as they are reported to be associated with the development of ischemic stroke [31]. This study was also done in Chinese patients and evaluated 301 patients with LAA stroke, with age- and sex-matched controls. A total of 1012 annotated CpG loci in 672 genes were identified as differentially methylated, based on the established threshold, which were involved in different aspects of the nervous system. The investigators looked particularly at the gene *MTRNR2L8*, that functions as a neuroprotective factor, and its promotor methylation status, and acknowledged its high predictive value in the prognosis of ischemic stroke, possibly serving as a guide to its prevention and clinical diagnosis [31].

In a genetic association study interleukin IL-6, IL-1β, monocyte chemoattractant protein-1 (CCL2) macrophage inflammatory protein-1α (CCL3), E-selectin (SELE), intercellular adhesion molecule 1 (ICAM1), and matrix metalloproteinase-3 (MMP-3), and 9 (MMP-9) gene variants were

found to be independently and significantly associated with atherosclerotic internal carotid stenosis [32].

3.6 Biomarkers found in retrieved thrombi

The advent of mechanical thrombectomy made possible the histologic analysis of retrieved thrombi to assess their origin. This was done by Wang *et al*, who studied thrombi in a prospective multicenter cross-sectional study of patients with ischemic stroke who had undergone thrombectomy. Even though it was a small study, with a total amount of 94 patients, of which 56 had a cardioembolic etiology and 36 an atherothrombotic, they found that thrombi with a LAA source had significantly higher actin and CD105 levels than thrombi with a cardioembolic source. The authors hypothesize that actin levels could predict a LAA source because of its association with plaque formation and stability [33]. An increased finding of a localized pattern of the oxidative stress marker 4-hydroxyl-2-nonenal has also been reported in clots retrieved from patients with LAA when compared to patients with cardioembolic and cryptogenic stroke (P 0 < 0.01) suggesting that it may be a novel marker of LAA [34].

4. Conclusions

Most blood biomarkers associated with LAA stoke evaluated patients' prognosis and the presence of vulnerable plaques. In the future, biomarkers could be used to stratify patients according to disease severity and to evaluate response to treatment. Overall, there is a preponderance of retrospective studies evaluating biomarkers associated with LAA stroke. Most studies evaluated Chinese patients and where not externally validated. RNA markers still need further studies to assess their clinical utility.

To adopt these biomarkers in clinical practice we will need robust multicentric studies proving their reproducibility and a clear practical applicability for their use.

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