Blood biomarkers to identify ischemic stroke etiologies

Stroke is one of the main causes of death and disability in the world. However, there are still several medical limitations in stroke prevention, diagnosis and treatment. One promising field under investigation is the use of biomarkers to guide stroke etiology diagnosis and classification, since treatment differs among etiologic subtypes (cardioembolic, atherosclerotic and lacunar stroke) and many patients currently receive a diagnosis of undetermined stroke. In this article we update current knowledge about biomarkers related to specific stroke etiologies and discuss whether using a combination of biomarkers seems a feasible strategy for making an etiologic diagnosis in the acute phase of stroke. In the future, the use of these biomarkers will allow clinicians to rapidly guide other diagnostic tests and accelerate the onset of an optimal secondary prevention.

KEYWORDS: atherosclerotic ■ biomarker ■ cardioembolic ■ classification ■ etiology ■ lacunar ■ stroke

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Stroke: 2010 situation

Stroke is the third leading cause of death and the most common cause of permanent disability in adults worldwide, carrying a significant socioeconomic burden for all countries [1].

Of all strokes, 87% are ischemic, 10% are intracerebral hemorrhages and 3% are subarachnoid hemorrhages [2]. At present, the diagnosis of stroke remains based on clinical grounds and neuroimaging assessment. As neuroimaging is not a widely available tool, other methods might be useful to differentiate between hemorrhagic and ischemic stroke or to discard pathologies that simulate cerebral ischemia at an early stage, mainly in the prehospital setting. Biomarkers use might aid in ruling out these 'mimics', such as tumors or epileptic seizures, that account for up to a third of patients with stroke-like symptoms [3], thus avoiding unnecessary urgent transfers, specialist evaluation, extra testing and improving the cost–effectiveness in the stroke field.

Intravenous thrombolysis with recombinant tissue-type plasminogen activator (rt-PA) currently remains the only approved acute treatment for ischemic stroke. However, despite great improvements in the field, very few stroke patients (1–5%) benefit from these therapies [4]. In this area, the applicability of biomarkers could help in improving safety and efficacy of the administration of reperfusion therapies.

Once the diagnosis and hyperacute treatment of ischemic stroke have been carried out, etiologic classification is critical to discern the best treatment offered both in the stroke units during the acute phase and at the outpatient clinics for the secondary prevention. However, even with a thorough evaluation, the etiology of ischemic stroke remains undetermined in 25–39% of patients [5]. The use of biomarkers for etiologic diagnostic assessment, the focus of this article, might contribute to the reduction of this percentage of cryptogenic strokes and to prescribe the most appropriate secondary treatments (anticoagulant or antiplatelet therapy).

Therefore, biomarkers in the neurovascular field would contribute in several steps, from evaluating stroke risk to predicting outcome or treatment response, improving the etiologic classification and subsequently the management of stroke patients, which makes personalized medicine in stroke an attractive scenario in the near future.

Stroke etiology classification systems

Given that stroke prognosis, risk of recurrence and choices for management greatly differ between stroke subtypes, several classification schemes have been developed since the 1970s in order to identify the most likely stroke etiology (**Table 1**) [5–14].

All of the classification systems have attempted to group patients into four to seven main categories, which might be further divided into different subcategories. Common problems with the eldest classification systems were the oversizing and heterogeneity of the undetermined category [6–8], which in most cases was the consequence of an incomplete evaluation of the patient. Later, new classifications,

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Table 1. Main stroke etiology classification systems.

Classification system	Date of publication	Main features	Categories	Comments	Ref.
Harvard Stroke Registry	1978	Only clinical	Five major etiologies: • Brain hemorrhages • Brain infarctions (atherothrombotic and tandem arterial pathological abnormalities, cardioembolic stroke, lacunar stroke, stroke from rare causes or undetermined etiologies)	Overestimation of 'undetermined etiology' (39%) Lack of diagnostic tools available when the classification was defined	[6,7]
Laussane Stroke Registry	1988	Clinical and diagnostic tests	Six categories: atherosclerosis with stenosis, atherosclerosis without stenosis, emboligenic heart disease, hypertensive arteriolopathy, mixed etiologies and other etiologies	Overestimation of atherosclerotic group	[12]
Oxfordshire Community Stroke Project	1991	Only clinical	Four main categories, according to the location: TACI, PACI, POCI and LACI	Predictive of outcome, not predictive of etiology	[13]
TOAST (Trial of Org 10172)	1993	Clinical plus other diagnostic tests (not required)	11 categories, five major etiologic groups: large-artery atherosclerosis, cardioembolism, small vessel occlusion, stroke of other determined causes and stroke of undetermined cause	Incomplete evaluation can be masked Heterogeneity and oversizing of the undetermined category	[8]
GENIC	2000	Clinical plus carotid ultrasonographic studies plus other diagnostic tests	Seven categories: atherothrombotic, cardioembolic, lacunar, arterial dissection, rare causes, undetermined causes and unknown causes	Overestimation of atherosclerotic group	[14]
SSS-TOAST	2005	Imaging proof of acute infarction is required, minimum level of diagnostic tests required	Each category of original TOAST subdivided into: 'evident', 'probable' and 'possible'	It decreases the number of patients with undetermined stroke, compared with the original TOAST	[9,10]
A-S-C-O	2009	Clinical and diagnostic tests	Four main categories: 'A', 'S', 'C' and 'O' Each phenotype is graded into: '1': definitely a potential cause for the index event, '2': causality uncertain and '3': unlikely a direct cause for the index event (but disease is present)	Etiologic classification of stroke, taking into account other underlying diseases Too many possible combinations	[5,11]

A: Atherosclerosis; C: Cardiac source; LACI: Lacunar infarction; O: Other causes; PACI: Partial anterior circulation infarction; POCI: Posterior circulation infarction; S: Small vessel disease; TACI: Total anterior circulation infarction.

particularly SSS-TOAST [9,10] and ASCO [5,11], have reduced the number of patients with undetermined etiology and accepted the existence of mixed phenotypes and underlying diseases associated with stroke.

In summary, although these classifications allow us to have a standard reference language, taken all together, they still have some important limitations. Moreover, these systems are based on associations because they lack a gold standard, such as pathological confirmation, to define the exact cause of stroke. Thus, we should take them only as the basis of our diagnosis skeleton; however, we need to develop other ancillary tests to make a correct etiologic classification. Here is where the stroke biomarkers may have an important future role if an etiologic 'biological signature' were to be studied in accessible body fluids.

Stroke biomarkers overview

The use of plasma biomarkers is getting increasingly popular in several fields of medicine. Interest in cerebrovascular diseases has also risen, since biomarkers might aid physicians in several steps of stroke evaluation, as we have discussed in the introduction.

At present, only some approaches using biomarkers to evaluate the risk of ischemic stroke appearance are close to being applicable to the clinical setting. That is the case of the recently US FDA approved blood test measuring lipoprotein-associated phospholipase A2, a circulating enzyme involved in inflammation that is an independent predictor of future stroke among healthy individuals [15]. Therefore, early stroke detection will permit physicians to prescribe lifestyle changes in order to reduce some risk factors or establish preventive treatments, such as lipid-lowering drugs.

Other investigations have tried to cover the necessity of ruling out mimics and having a prompt biochemical diagnosis for ischemic stroke with the use of biomarkers, individually (S100B, neuron-specific enolase, NMDA receptor peptides or antibodies, RNA binding protein regulatory subunit [PARK7], nucleotide diphosphate kinase A) or combining them in different panels. Regrettably, none of these studies have demonstrated enough success [16].

Once the diagnosis of stroke has been made, a guide to evaluate the stroke outcome and progression would be useful to optimize inpatient care [17]. Prognostic biomarkers might identify those patients that are going to ameliorate, to remain stable or to suffer several complications, thus helping in decision-making regarding the use of specialized stroke units. These biomarkers must add information to validated clinical scales and improve their predictive power.

As an example, one of the most feared complication of thrombolytic therapy is symptomatic intracranial hemorrhage (SICH), affecting 3–10% of rt-PA-treated patients [18]. For predicting which patients will suffer a SICH and then have a poor outcome, matrix metalloproteinase (MMP)-9 might be a promising biomarker as this enzyme could have a role in blood–brain barrier dysfunction. In addition, another molecule that might be indicative of this endothelial damage is cellular fibronectin (c-Fn). According to that hypothesis, baseline MMP-9 and c-Fn plasma levels have been associated with the different degrees of hemorrhagic transformation after rt-PA administration [19,20]. In this case, biomarkers may assist in the management of patients with a higher risk of SICH; however, MMP-9 and c-Fn sensitivity and specificity is not high enough to be used in taking clinical decisions such as whether to give rt-PA to an individual stroke patient or not.

Finally, although there is currently no single biomarker approved to identify stroke etiology, several promising candidates have recently been described. In the next part of this article, we summarize the studies that have tried to differentiate stroke etiologies by using different blood biomarkers.

Biomarkers to identify stroke etiologies

According to the most popular classification system for stroke etiology, the TOAST classification [8], three main causes of ischemic stroke can be determined: large-artery atherosclerosis (LAA), cardioembolism (CE) and small vessel disease (SVD) (Table 1). Patients suffering from stroke in each of these etiologies are managed in a different way. On the one hand, if the thrombus has been formed within the process of atherosclerotic plaque rupture, then the secondary prevention treatment more suitable is antiplatelet therapy and, in selected cases, surgery (e.g., carotid endarterectomy). By contrast, when a CE stroke occurs (i.e., when a blood clot is formed on the heart and carried by the bloodstream to occlude a brain artery), it is usually treated with anticoagulant drugs. In the case of a lacunar stroke, the secondary prevention is carried out by means of antiplatelet agents or antihypertensive drugs. For this reason, it would be

very useful to have a complementary diagnostic tool that could specifically define which etiology is involved in a stroke event in order to provide the optimal treatment.

Otherwise well-validated etiological biomarkers would help to elucidate which is the essential cause in those strokes of 'undetermined etiology' and thus optimizing the treatment [21,22].

One limitation in the identification of etiological biomarkers is that studies start from a previous clinical etiology classification (i.e., the biological samples of patients used for biomarkers research have been previously classified as LAA, CE or SVD strokes and the biomarkers discovered will be assigned to that clinical etiology). Moreover, in each particular study the etiology classification system used is chosen by the investigators, since there is more than one possibility. In these studies the major difficulty is the absence of a diagnostic standard for cryptogenic strokes; therefore, the research conducted to identify ideal biomarkers for each stroke subtype would be carried out in stroke cases with a very well-defined etiological phenotype.

Current research is mainly being carried out in blood biomarkers, since in future clinic applications blood tests will be an accessible tool. In this section, we review the current knowledge regarding proteins, mRNA and polymorphisms studied as biomarkers of stroke etiology. Apart from these molecules, other types of biological elements could be indicative of processes related to the stroke pathology and they need to be taken into account, such as protein fragments (e.g., D-dimer), peptides (e.g., telopeptide from collagen), amino acids (e.g., asymmetric dimethylarginine), lipids (e.g., ceramide) or blood particles (e.g., platelet-derived microparticles). All of these biomarkers are listed in **Tables 2, 3 & 4** [21–84] according to the etiology they are related to (CE, LAA or SVD, respectively).

Moreover, we have also included some candidates that despite having never been tested to identify stroke etiologies are involved in some diseases considered as risk factors for stroke or endophenotypes, such as atrial fibrillation (AF) for CE stroke or intima-media thickness (IMT) and the metabolic syndrome for LAA stroke. We should emphasize that these candidates will not necessarily be biomarkers of such stroke etiology, but among them scientists might discover some promising biomarkers for stroke subtypes. Future studies involving those biomarkers should include multivariate statistical analysis to correct for confounding factors, such as associations due to underlying pathologies.

Plasma proteins related to stroke etiology

The ideal candidates should be related to the main processes involved in stroke pathophysiology such as hemostasis, inflammation, immune system activation, endothelial damage or oxidative stress.

Abnormalities of coagulation and fibrinolysis may play an important role in the pathogenesis of ischemic stroke. Thus, molecules involved in hemostasis might be useful biomarkers. For example, fibrinopeptide A and prothrombin fragment, that reflect thrombin activity, or D-dimer, a product of fibrin degradation, appear in the circulation when the coagulation system has been activated and red fibrin-rich thrombi have been formed. Those red clots typically originate in diseased cardiac chambers [23] and are related to CE stroke etiology (**Table 2**) [21,24]. In fact, Ageno and colleagues [25] described D-dimer as a marker of CE stroke in a cohort of 126 patients, with a predictive cutoff point for CE stroke of 2.00 µg/ml, resulting in a specificity of 93.2% and a sensitivity of 59.3%.

In the largest study ever conducted on biomarkers and stroke etiology, high levels of B-type natriuretic peptide (BNP; a cardiac hormone with an antifibrotic role in the heart) and D-dimer predicted CE stroke in 707 ischemic stroke patients [21]. BNP over 76 pg/ml (odds ratio [OR]: 2.3; 95% CI: 1.4–3.7; p = 0.001) and D-dimer over 0.96 µg/ml (OR: 2.2; 95% CI: 1.4–3.7; p = 0.001) were independent predictors of CE stroke and even among patients with transient symptoms (n = 155), a high BNP level identified CE etiology (OR: 6.7; 95% CI: 2.4–18.9; p < 0.001). In this study, a model combining clinical and biochemical data had a sensitivity of 66.5% and a specificity of 91.3% for predicting CE. A practical example of the use of that model is illustrated in **Figure 1**, showing how combining biomarkers might improve the diagnosis of the etiology of stroke, mainly in undetermined cases.

This was recently confirmed by Shibazaki and colleagues [26] who described an optimal cutoff of plasma BNP levels of 140.0 pg/ml to distinguish CE from other stroke subtypes. This was extended to proform BNP by Rodríguez-Yáñez and collaborators [22], who studied 262 patients with first ischemic stroke within the first 12 h and showed that a proform BNP level over 360 pg/ml was independently associated with CE stroke (OR: 28.51; 95% CI: 5.90–136.75; p < 0.0001).

Table 2. Candidate markers of cardioembolic stroke.

Name	Type of biomarker	Accession # for proteins	Reason for being a candidate	Ref.
Biomarkers that have been assayed in stroke patients				
ANP	Protein	P01160	Marker of cardiac damage. Higher levels in CE stroke versus other etiologies (262 patients)	[22]
BNP	Protein	P16860	Higher plasma levels in patients suffering from acute CE strokes versus other etiologies. Independent predictor of CE stroke	[21,26–28]
Proform BNP	Protein	P16860	Higher levels in CE stroke patients versus other etiologies (262 patients). Pro-BNP might be useful to reclassify undetermined stroke as of CE origin	[22]
sCD40L†	Protein	P29965	Inflammatory marker. Elevated in AF patients. No differencies found among etiologies within 107 acute ischemic stroke patients	[29,30]
CRP†	Protein	P02741	Risk marker for AF. Increased in acute CE stroke versus other etiologies (648 patients studied)	[24,31]
D-dimer	Protein fragment		Breakdown product of fibrin, elevated in patients with AF. Independent predictor of CE stroke (707 ischemic stroke patients)	[21,24,25]
IL-1β	Protein	P01584	Higher levels in subacute CE stroke versus other etiologies (120 patients involved)	[32]
IL-6	Protein	P05231	Proinflammatory cytokine. Elevated in AF patients and in subacute CE stroke versus other etiologies (120 patients studied)	[24,32,33]
PITX2	Gene	Q99697	Transcription factor associated with AF. Gene polymorphism associated with CE stroke	[34,35]
TNF-α	Protein	P01375	Proinflammatory cytokine. Elevated in AF patients and in subacute CE stroke versus other etiologies (120 patients studied)	[24,32]
ZFHX3	Gene	Q15911	Gene polymorphism associated with AF and CE stroke	[36]
Possible candidates based on underlying pathological risk of cardioembolic stroke				
Adiponectin	Protein	Q15848	Elevated in patients with persistent AF	[37]
Ang-2	Protein	O15123	Angiogenic factor. Elevated in AF patients	[29]
Apelin	Protein	Q9ULZ1	It seems to be involved in regulation of the angiotensin/vasopressin system. Lower levels in lone AF patients, correlating negatively with pro-BNP	[38]
ADMA	Amino acid		Endogen inhibitor of NOS. ADMA contributes to the thromboembolism in AF	[39]
CITP	Peptide		Marker of collagen degradation. Elevated in patients with AF	[37,40]
CD63†	Protein	P08962	Platelet activation marker. More positive cells in AF patients (121 patients studied)	[41]
Coagulation factor VII (FVII)	Gene	P08709	A promoter polymorphism is associated with risk reduction of CE stroke in AF patients	[42]

†*Candidate markers with controversial results among studies.*

ADMA: Asymmetric dimethylarginine; AF: Atrial fibrillation; Ang: Angiopoietin; ANP: Atrial natriuretic peptide; BNP: B-type natriuretic peptide; CE: Cardioembolism; CITP: Carboxy-terminal telopeptide of collagen type I; CRP: C-reactive protein; MMP: Matrix metalloproteinase; NOS: Nitric oxide synthase; NPY: Neuropeptide Y; PF: Platelet factor; PITX2: Paired-like homeodomain; PMP: Platelet microparticle; pro-BNP: Proform B-type natriuretic peptide; sCD40L: CD40 ligand, soluble; TF: Tissue factor; TIMP: Tissue inhibitor of metalloproteinase; vWF: von Willebrand factor; ZFHX3: Zinc finger homeobox 3.

Table 2. Candidate markers of cardioembolic stroke (cont.).

Name	Type of biomarker	Accession # for proteins	Reason for being a candidate	Ref.
Possible candidates based on underlying pathological risk of cardioembolic stroke (cont.)				
E-selectin, soluble	Protein	P16581	Marker of endothelial activation. Elevated in AF patients	[43]
Fibrinopeptide A	Protein	P02671	Increased in patients with AF. It reflects thrombin activity	[24]
MMP-1	Protein	P03956	Patients with AF had MMP-1 reduced	[44]
MMP-9	Protein	P14780	Elevated in AF patients versus controls	[45]
NPY	Protein	P01303	Elevated in AF patients versus controls	[45]
PF-4	Protein	P02776	Marker of platelet activation in AF patients	[24]
PMP	Blood particles		Higher number of particles in AF than in healthy controls	[46]
Prothrombin fragment 1.2 (F1+2)	Protein fragment		Marker of thrombogenesis. Increased in AF patients	[44]
P-selectin (CD62P)†	Protein	P16109	More positive cells in AF patients with high risk of stroke	[41]
P-selectin, soluble	Protein	P16109	Marker of platelet activation in AF patients	[24,41]
TF	Protein	P13726	Coagulation. Higher levels in AF patients	[24]
TIMP-1	Protein	P01033	Increased in AF patients	[44]
TGF-β	Protein	P01137	Profibrotic cytokine. Higher levels predict persistent AF	[47]
VEGF	Protein	P15692	Elevated in AF patients	[29]
vWF	Protein	P04275	Marker of endothelial damage/dysfunction. Higher plasma levels in AF patients, where predicts cardiovascular events	[24,48]

†*Candidate markers with controversial results among studies.*
ADMA: Asymmetric dimethylarginine; AF: Atrial fibrillation; Ang: Angiopoietin; ANP: Atrial natriuretic peptide; BNP: B-type natriuretic peptide; CE: Cardioembolism; CITP: Carboxy-terminal telopeptide of collagen type I; CRP: C-reactive protein; MMP: Matrix metalloproteinase; NOS: Nitric oxide synthase; NPY: Neuropeptide Y; PF: Platelet factor; PITX2: Paired-like homeodomain; PMP: Platelet microparticle; pro-BNP: Proform B-type natriuretic peptide; sCD40L: CD40 ligand, soluble; TF: Tissue factor; TIMP: Tissue inhibitor of metalloproteinase; vWF: von Willebrand factor; ZFHX3: Zinc finger homeobox 3.

Table 3. Candidate markers of large-artery atherosclerotic stroke.

Name	Type of biomarker	Accession # for proteins	Reason for being a candidate	Ref.
Biomarkers that have been assayed in stroke patients				
15-d-PGJ2	Lipid		Higher levels in LAA versus other etiologies of stroke (552 acute ischemic stroke patients)	[49]
CCR5, RANTES	RNA	P13501	Gene expression higher in LAA versus CE stroke	[23]
sCD40L†	Protein	P29965	Marker of platelet activation associated with proinflammatory and proatherogenic effects. Higher levels in acute coronary syndrome patients. No differencies found among etiologies within 107 acute ischemic stroke patients	[30,50,51]
CD63†	Protein	P08962	Platelet activation marker. More positive cells in patients suffering from subacute LAA stroke versus SVD (80 patients)	[52]
Fetuin-A†	Protein	P02765	Proinflammatory effects, inhibition of adiponectin, correlation with insulin resistance and with carotid arterial stiffness. Higher levels predict risk of stroke. No differencies found among etiologies within 107 acute ischemic stroke patients	[30,53–55]
GGT	Protein	P19440	GGT presence in atherosclerotic plaques. Higher levels in acute non-CE ischemic stroke patients, independently of risk factors	[56]
Hp	Protein	P00737	Higher serum levels in acute LAA stroke versus other etiologies (262 patients). Hp was found expressed only in symptomatic plaques	[57]
Lp(a)	Protein	P08519	Procoagulant protein. Elevated in LAA versus other etiologies of stroke (253 patients)	[58]
Lp-PLA2	Protein	P04054	Proinflammatory role. Higher risk of recurrence in patients that had suffered from an LAA stroke than other etiologies (467 patients studied)	[59]
PPBP	RNA	P02775	Gene expression higher in LAA versus CE stroke	[23]
PDGFA	RNA	P04085	Gene expression higher in LAA versus CE stroke	[23]
PF-4	RNA	P02776	Gene expression higher in LAA versus CE stroke	[23]
Protein Z	Protein	P22891	It seems to inhibit thrombus formation. Higher plasma levels in the subacute phase of LAA stroke versus other etiologies (173 patients)	[60]
P-selectin (CD62P)†	Protein	P16109	Platelet activation marker. More positive cells in patients suffering from subacute LAA stroke versus SVD (80 patients)	[52]
SCUBE1	Protein	A0JP65	Marker of platelet activation. Higher levels in acute coronary syndrome and in LAA versus SVD strokes	[50]
SAA	Protein	P02735	Inflammatory marker. Higher serum levels in acute LAA stroke versus other etiologies (262 patients). SAA might be useful to reclassify undetermined stroke as of LAA origin	[57]

†*Candidate markers with controversial results among studies.*

15-d-PGJ2: 15-deoxy-δ prostaglandin J2; ACE: Angiotensin converting enzyme; CAM: Cell adhesion molecule; CCL: C-C motif chemokine; CCR5: C-C motif chemokine 5; CE: Chemoembolism; CRP: C-reactive protein; GGT: γ-glutamyltransferase; HDL: High-density lipoprotein; Hp: Haptoglobin; IMT: Intima-media thickness; LAA: Large-artery atherosclerosis; LDL: Low-density lipoprotein; Lp: Lipoprotein; MCP: Monocyte chemotactic protein; MCSF: Macrophage colony-stimulating factor; MMP: Matrix metalloproteinase; MPO: Myeloperoxidase; oxLDL: Oxidized low-density lipoprotein; PAI: Plasminogen activator inhibitor; PAPP-A: Pregnancy-associated plasma protein A; PF: Platelet factor; PLA2: Phospholipase A2; PPBP: Platelet basic protein; RAGE: Receptor for advanced glycosylation end product; SAA: Serum amyloid A protein; sCD40L: CD40 ligand, soluble; SVD: Small vessel disease.

Table 3. Candidate markers of large-artery atherosclerotic stroke (cont.).

Name	Type of biomarker	Accession # for proteins	Reason for being a candidate	Ref.
Possible candidates based on underlying pathological risk of atherosclerotic stroke				
Adiponectin	Protein	P02741	Antiatherogenic properties. Lower levels in intracranial atherosclerosis and as a risk factor for early atherosclerosis	[61,62]
ACE	Gene	P12821	*D* allele polymorphism associated with increased IMT	[63]
APO A-I	Protein	P02647	Major component of HDL. Atheroprotective	[64]
APO B	Protein	P04114	Major component of LDL. Atherogenic	[64]
APO E	Gene	P02649	*ε4* allele associated with increased IMT, but *ε2* allele associated with reduced IMT	[65,66]
Ceramide	Lipid		Lipoprotein aggregation product, 10- to 50-fold higher in LDL from plaques than from plasma. Proinflammatory effect by means of CRP and IL-6	[67]
CRP†	Protein	P02741	It enhances endothelial permeability, CAM expression, platelet activation and thrombogenesis. Involved in foam cell formation	[51,68]
MCSF	Protein	P09603	Proinflammatory cytokine. Involved in foam cell formation	[69]
MMP-9	Protein	P14780	Elevated in unstable plaques. Statins reduce MMP-9 levels	[70]
MCP-1; CCL2	Protein	P13500	It is present in macrophage-rich atherosclerotic plaques. oxLDL induce production of MCP-1	[71]
MPO	Protein	P05164	Proatherogenic effect	[51,72]
Oxidized phospholipids	Lipid		They are involved in plaque rupture	[72]
PAI-1	Protein	P05121	Marker of endothelial disfunction and atherosclerosis progression	[68]
Platelet glycoprotein 4 (CD36)	Protein	P16671	Involved in development of foam cells and platelet activation. Higher plasma levels in patients with symptomatic atherosclerotic carotid plaques	[73]
PAPP-A	Protein	Q13219	Expressed in unstable plaques, but not in stable ones	[51]
RAGE	Protein	Q15109	It acts as an amplifier of the inflammatory signal. In addition, its ligand interaction leads to changes in the dynamic properties of endothelial cells	[74]

†*Candidate markers with controversial results among studies.*

15-d-PGJ2: 15-deoxy-δ prostaglandin J2; ACE: Angiotensin converting enzyme; CAM: Cell adhesion molecule; CCL: C-C motif chemokine; CCR5: C-C motif chemokine 5; CE: Chemoembolism; CRP: C-reactive protein; GGT: γ-glutamyltransferase; HDL: High-density lipoprotein; Hp: Haptoglobin; IMT: Intima-media thickness; LAA: Large-artery atherosclerosis; LDL: Low-density lipoprotein; Lp: Lipoprotein; MCP: Monocyte chemotactic protein; MCSF: Macrophage colony-stimulating factor; MMP: Matrix metalloproteinase; MPO: Myeloperoxidase; oxLDL: Oxidized low-density lipoprotein; PAI: Plasminogen activator inhibitor; PAPP-A: Pregnancy-associated plasma protein A; PF: Platelet factor; PLA2: Phospholipase A2; PPBP: Platelet basic protein; RAGE: Receptor for advanced glycosylation end product; SAA: Serum amyloid A protein; sCD40L: CD40 ligand, soluble; SVD: Small vessel disease.

Table 4. Candidate markers of lacunar stroke.

Name	Type of biomarker	Accession # for proteins	Reason for being a candidate	Ref.
Biomarkers that have been assayed in stroke patients				
Hp	Gene	P00737	Hp1–1 phenotype has been associated with SVD stroke patients in the chronic stage (124 patients included in the study)	[75]
Hcy	Amino acid		Involved in vascular damage. High levels of Hcy associated with SVD stroke in different population studies including all etiologies. High levels also correlate with leukoaraiosis severity	[76–78]
IL-1β	Protein	P01584	Proinflammatory cytokine. Lower levels in the subacute phase of SVD stroke versus other etiologies (120 patients studied)	[32]
IL-6	Protein	P05231	Proinflammatory cytokine. Lower levels in the subacute phase of SVD stroke versus other etiologies (120 patients studied)	[32]
PRKCH	Gene	P24723	Gene nonsynonymus SNP associated with SVD stroke	[79]
TNF-α	Protein	P01375	Proinflammatory cytokine. Lower levels in the subacute phase of SVD stroke verus other etiologies (120 patients studied)	[32]
Possible candidates based on underlying pathological risk of lacunar strokes				
Adiponectin	Protein	Q15848	Lower levels in untreated hypertensive women levels versus controls	[80]
E-selectin, soluble	Protein	P16581	Endothelial dysfunction marker. Increased serum levels in patients with impaired vasodilation	[81]
IGF-1	Protein	P01343	Low levels of this hormone (<79.4 μg/l) are associated with cognitive decline in hypertensive elderly subjects	[82]
MMP-9	Protein	P14780	High plasma MMP-9 level predicts a 1.97-fold increased risk of blood pressure progression in 595 nonhypertensive people at 4-year follow-up. Increased levels in normotensive individuals with family history of hypertension	[83,84]
MCP-1; CCL2	Protein	P13500	Biomarker of inflammation. Increased serum levels in patients with impaired vasodilation	[81]
P-selectin, soluble	Protein	P16109	Endothelial dysfunction marker. Increased serum levels in patients with impaired vasodilation and in normotensive individuals with family history of hypertension	[81,84]
RBP4	Protein	P02753	Adipocytokine found elevated in untreated hypertensive women, with strong correlation with IMT	[80]
TIMP-1	Protein	P01033	High plasma TIMP-1 level predicts a 2.15-fold increased risk of hypertension in 595 nonhypertensive people at 4-year follow-up. Increased serum levels in patients with impaired vasodilation and in normotensive individuals with family history of hypertension	[81,83,84]

CCL: C-C motif chemokine; Hcy: Homocysteine; Hp: Haptoglobin; IMT: Intima-media thickness; MCP: Monocyte chemotactic protein; MMP: Matrix metalloproteinase; PRKCH: Protein kinase C, eta; RBP: Retinol-binding protein; SNP: Single-nucleotide polymorphism; SVD: Small vessel disease; TIMP: Tissue inhibitor of metalloproteinase.

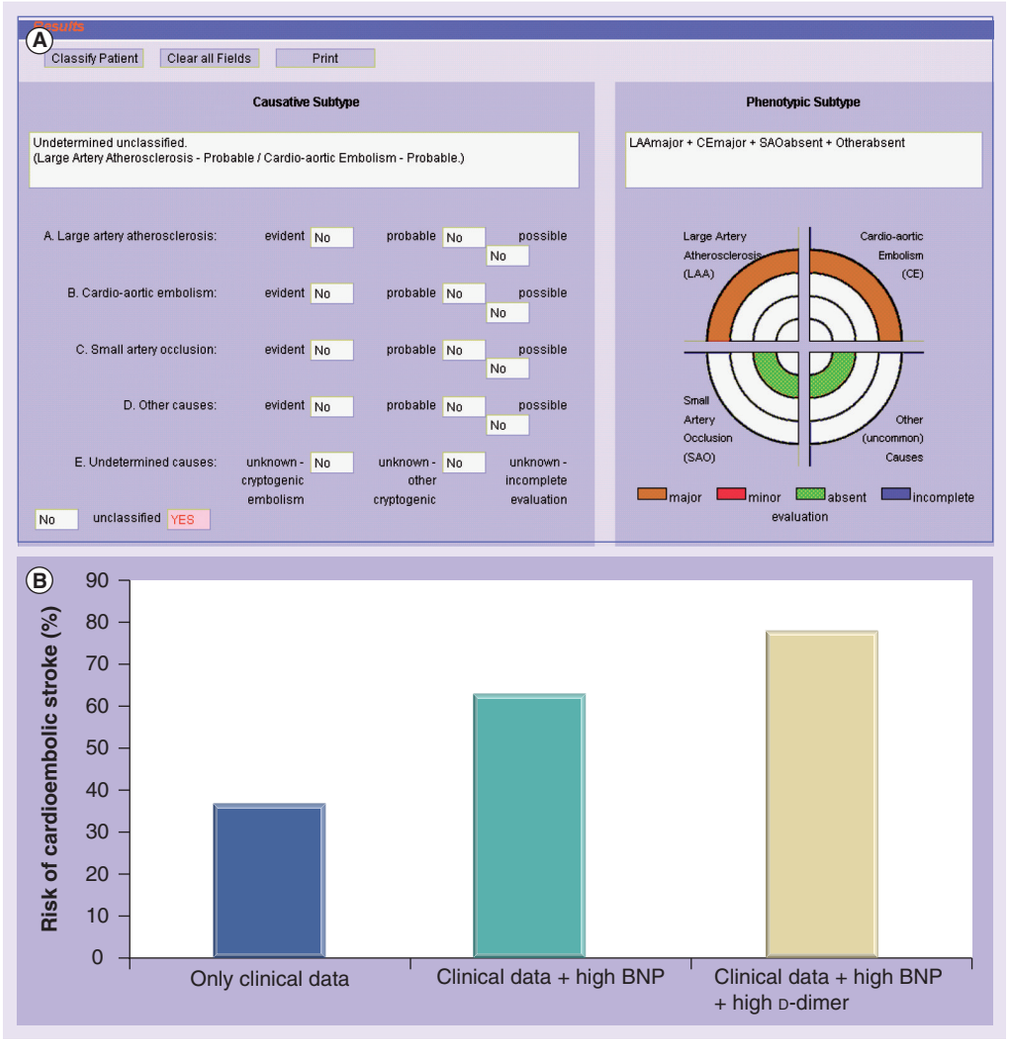

Figure 1. Biomarkers use in the diagnosis of stroke etiology. (A) Visual output from the Causative Classification System (CCS) [201] in a hypothetical case of a 74-year-old male patient who was a smoker with a past history of hypertension and an acute stroke (NIHSS = 7) without demonstration of a lesion in the neuroimaging that might be related to two causes: atrial fibrillation and/or severe carotid stenosis. **(B)** Applying the predictive model described by Montaner and colleagues [21] we increased the risk of cardioembolic stroke from 37 to 78% in the presence of two high cardioembolic stroke-related biomarkers (BNP and D-dimer).
BNP: B-type natriuretic peptide.

Moreover, as white platelet-rich thrombi also exist, platelet activation is another phenomenon that could provide some good candidates such as CD40 ligand (CD40L), CD63 or P-selectin, which are mainly associated with LAA strokes, although these biomarkers have only been compared among non-CE strokes [50,52] (Table 3). However, some investigators have found these markers to be elevated in AF patients [29,41], which reveals the necessity of studies to include all stroke etiologies.

Plasma levels of inflammatory markers in acute ischemic strokes were correlated with the TOAST subtype in a recent study by Licata and colleagues [32], showing how different the proinflammatory cytokines profile is between etiologies. In their study, IL-1β, IL-6 and TNF-α were elevated in CE strokes compared with other etiologies (Table 2). Otherwise, these cytokines would have had lower levels in SVD stroke when compared with all other stroke etiologies (Table 4). The authors suggested that this might be explained by the different parenchymal zones affected by the infarct and, consequently, by the different cellular components involved.

This possible association between CE stroke and inflammation is suggested by several studies based on the identification of inflammatory serum biomarkers that are elevated in patients with AF (a typical source of embolism). In this

population, the successfulness of sinus rhythm maintenance after cardioversion and the risk of CE stroke are related to the inflammatory burden [24,33]. Indeed, inflammation and thrombosis are intimately related, and an association between AF and TNF-α has been demonstrated; moreover, patients with AF have higher levels of IL-6, plasma viscosity and tissue factor, with the later relationship being maintained even after adjustment for confounding factors [24].

In this line of evidence, one of the most studied proteins is C-reactive protein (CRP), an acute-phase reactant protein that increases in response to different stimuli, such as inflammation or infection. It increases within 6 h and has a peak at approximately 50 h after stimulus [85]. CRP has been associated with LAA events (Table 3), on account of its involvement in the inflammatory process, platelet activation and macrophages differentiation to foam cells [51,68]. However, Terruzzi and colleagues recently analyzed CRP plasma levels within the 6 first h after stroke onset and showed higher levels in the CE subset than in other etiologies (Table 2) [31]. The increase of plasma CRP could be explained by the inflammatory environment in heart diseases such as AF, as we have shown previously. Another plausible explanation is that CRP is elevated because of the common largest extension of brain injury and severity usually seen in CE strokes, as higher CRP levels have been associated with stroke severity and mortality [86,87].

Nevertheless, some inflammatory markers have been associated with LAA stroke (Table 3), such as lipoprotein A that was evaluated in 253 consecutive patients with acute ischemic stroke in whom lipoprotein A levels over 30 mg/dl were more frequent among the LAA subgroup than among the CE subgroup (39.4 vs 11.1%; $p < 0.001$) [58]. Two other proteins related with the inflammatory process were found associated with LAA etiology when compared with the others in a proteomic study of serum samples: haptoglobin and serum amyloid A. These proteomic results were confirmed in a larger series of ischemic stroke patients (n = 262) using enzyme-linked immunosorbent assay techniques, where haptoglobin levels over 1040 µg/ml identified LAA patients with 95% sensitivity and 88% specificity, whereas serum amyloid A levels over 160 µg/ml identified LAA patients with 91% sensitivity and 83% specificity [57].

The same group of investigators also studied 15-deoxy-δ prostaglandin J2, a natural anti-inflammatory prostaglandin, in 552 patients with acute stroke [49]. Levels of this biomarker were also significantly higher in patients with vascular risk factors (history of hypertension or diabetes) and with LAA infarcts (113.9 pg/ml [95% CI: 81.6–139.7]) than in those with SVD (58.7 pg/ml [95% CI: 32.7–86.2]), CE (12.1 pg/ml [95% CI: 6.5–39.2]) or undetermined origin infarcts (11.4 pg/ml [95% CI: 5.6–24.3]); $p < 0.0001$. The increase in 15-deoxy-δ prostaglandin J2 might be explained as a compensatory effect of the inflammatory burden in stroke.

Very recently, high levels of inflammatory markers shared by both LAA and CE strokes such as CD40L and fetuin-A have been described [30], showing that inflammation may be a general phenomenon in stroke, although more investigation needs to be carried out.

Regarding biomarkers that could diagnose SVD etiology of stroke, one of the most promising is homocysteine (Hcy), an amino acid involved in vascular damage.

Various studies have shown that Hcy levels are higher in SVD strokes than in the other etiologies [76–78]. The first of these studies analyzed the levels of Hcy in 775 acute ischemic strokes, showing that the highest quartile (>18.6 mmol/l) had the highest OR adjusted for SVD patients [76]. This result has been validated by recent investigations carried out in Black [77] and Asian populations [78]; moreover, in the recent study, levels of Hcy also correlated with leukoaraiosis severity.

Some of the explored pathways reflect the complexity of finding good candidate biomarkers to identify the different etiologies of ischemic stroke, as some results were conflicting. Moreover, the characteristics of the different studies performed (e.g., different cohorts, different end points, different analytical conditions and patients' treatments) make it hard to reach a consensus regarding the most suitable biomarkers involved in each etiology. In fact, some etiologies may share mechanisms (and therefore biomarkers) leading to stroke, such as a coronary disease that used to be atherothrombotic but may produce a cardiac discinesia or other heart disfunctions that generate a CE stroke. The measurement of those markers in different time points (acute vs subacute or chronic stages of neurovascular disease) also includes some other bias in these studies.

To overcome all of these limitations, more studies involving large clinical series to compare the biomarkers among all etiologies should be carried out in the near future and it is likely that well-designed diagnostic trials will lead to clinical validation of selected stroke biomarkers.

Another approach that should be adopted is to test panels of biomarkers that had previously been related with a specific etiology in individual studies, in order to find a combination that would permit etiological diagnosis, maybe using markers for the different processes involved (e.g., inflammation, endothelial damage and hemostasis).

Technically, the time taken to obtain results may vary, so this needs to be taken into account; ideally, information would be gained within hours or days during the acute subacute phase – this positions proteins at the best place to become etiology biomarkers.

Differential gene expression among stroke etiologies

Although blood proteins have been the main molecules evaluated as biomarkers in recent years, lately some investigations support the usefulness of nucleic acids in the diagnosis of stroke. In 2008, Xu and colleagues presented the first study showing differences of gene expression when they compared CE with LAA strokes [23]. Using RNA microarrays technology, they found 23 genes that can differentiate both etiologies with a high grade of specificity/sensitivity. Patients suffering a LAA stroke showed an inflammatory profile, since they had upregulated genes expressed in platelets (e.g., chemokines: pro-platelet basic protein gene [*PPBP*], platelet factor-4 gene [*PF4*], platelet-derived growth factor-α gene [*PDGFA*] and C-C motif chemokine 5 gene [*CCR5*]) and monocytes, both of which are involved in the development of atherosclerotic plaques and according to the most suitable antiplatelet treatment. On the other hand, genes upregulated in the CE etiology were expressed in neutrophils (peptidase inhibitor 3 gene [*PI3*]) and modulate the immune response (e.g., orosomucoid 1 gene [*ORM1*] or poliovirus-receptor related 2 gene [*PVRL2*]).

Another approach to using nucleic acids as biomarkers is by studying the miRNA, small noncoding RNAs, which control gene expression by both inhibition and activation. In a young Asian cohort, when comparing the expression profile of 836 miRNAs among CE, SVD and LAA strokes using microarrays, it was found that 132 miRNAs were useful in predicting etiology [88]. The miRNAs regulated were involved in endothelial/vascular function, angiogenesis, hematopoietic regulation and immune response.

Even though there are few reports in this field, the promising results obtained by means of these screening techniques using arrays should be analyzed with more detail in the future to find some combination of genes or changes in gene expression that could characterize each stroke etiology. Furthermore, these studies demonstrate how different the scenarios of the stroke pathophysiology are among etiologies and also which processes are altered depending on the cause of stroke.

Specific gene polymorphisms of each stroke etiology

At present, there are not many studies trying to identify genetic markers of etiology in ischemic stroke. A few analyses have attempted to find these biomarkers but with poor results [89] or with a limited sample size. The great majority of studies have focused on finding genetic risk factors for the different etiologies. In addition, the studies were focused on candidate gene approach, with no validation by other independent studies.

Until now, the most promising results have come from studies in CE stroke and used genome-wide analysis (GWA) approaches. Several studies using GWAs that detect over 1 million single-nucleotide polymorphisms (SNPs) across the genome found significant signals in CE stroke [35,36]. All of these studies have identified SNPs that are also associated with AF, the main risk factor for CE stroke. The DeCODE group found a SNP in chromosome 4q25 (rs2200733, near paired-like homeodomain gene [*PITX2*]) associated with ischemic stroke but only in the CE etiology. This gene encodes a catenin-regulated transcription factor associated with AF [34]. In this analysis, the DeCODE group genotyped 1661 ischemic stroke cases and 10,815 controls using the Infinium HumanHap300 chip that detects over 300,000 SNPs. The most significant SNPs were replicated in two new different cohorts of 2224 cases and 2583 controls and in 2327 cases and 16,760 controls. After the two replications were carried out, only rs2200733 was statistically associated with CE stroke [35].

Very recently, a SNP in the zinc finger homeobox 3 gene (*ZFHX3*) that encodes a homeodomain zinc-finger protein has been associated with AF and CE stroke [36]. The rs7193343 variant of *ZFHX3* was found after the analysis of 2385 AF cases and 33,752 controls. In a second phase analysis, the SNP was genotyped in five ischemic case–control sample sets of European descent comprising 1036 cases and 3468 controls and reached significant association.

In the case of LAA strokes all the positive studies published are based in candidate genes analysis. IMT is an intermediate phenotype strongly associated with LAA stroke and in fact the most interesting studies in LAA stroke have come from those that have been focused on the

IMT phenotype. Several candidate genes have been replicated and validated by independent studies [63,65,66,90,91]. These candidate genes can be classified into the following groups: lipid metabolism, inflammation, extracellular matrix, renin–angiotensin system, glucose metabolism, endothelial function and vasomotor regulation. Until recently, the genes most extensively studied were *APOE*ε polymorphisms and angiotensin converting enzyme (*ACE*) *ID insertion/deletion* polymorphism.

The *APOE*ε polymorphism has three common alleles (ε*2*, ε*3* and ε*4*) that produce three protein isoforms. Two recent large studies with a combined sample size of more than 18,000 subjects [65,66] have indicated that the ε*4* allele, associated with higher cholesterol levels and Alzheimer's disease [92,93], is associated with increased IMT, whereas the ε*2* allele is associated with decreased IMT.

The ACE converts angiotensin I to the strong vasoconstrictor angiotensin II, leading, among other effects, to an increased vascular tone and neointimal proliferation [90,91]. ACE has been involved in hypertension. The *D* allele has an additive effect on elevating plasma levels of ACE [94]. A meta-analysis that included 9833 subjects from 23 studies demonstrated that the *D* allele was associated with increased IMT [63].

Genome-wide linkage studies in the Icelandic population found that the phosphodiesterase 4D gene (*PDE4D*) was associated with ischemic stroke and particularly with LAA subtype [95], but further studies and meta-analysis have confirmed that this association is consistent only in Iceland and not in other populations [96]. Other important GWAs performed in ischemic stroke found an association of two SNPs near the ninjurin 2 gene (*NINJ2*) associated with ischemic stroke [97]. These associations were stronger in the LAA group, regrettably these associations have not been replicated in a well-powered study performed recently, indicating that perhaps these SNPs are false positives [98].

Small vessel disease stroke is associated with the monogenic forms of stroke caused by mutations in a unique gene. This is the case of CADASIL, the most frequent form of monogenic stroke that causes recurrent lacunar strokes. However, only several studies have been performed in SVD stroke with correct replication and validation of the results. The most important study was carried out in the Japanese population using 52,608 SNPs and replicated in two new cohorts [80]. A nonsynonymous SNP in the protein kinase C η gene (*PRKCH*) that affects protein kinase activity was found to be associated with SVD stroke. More studies have been performed in SVD stroke following a strategy of candidate genes approach [99,100]. The genes that belong to the renin–angiotensin system have been analyzed in different studies but without definitive results.

In conclusion, there are not specifically designed studies that have been developed to find genetic differences between the etiologies of ischemic stroke; however, the second wave of GWAs in ischemic stroke that will be carried out next year might find these genetic differences through the promising use of endophenotypes. It is remarkable that genetic biomarkers should not be standalone diagnostic biomarkers, as their existence is present before the stroke occurrence and their effect depends greatly on environmental and behavioral factors. However, genetic factors could predispose to conventional risk factors and this predisposition may differ between etiologic subtypes of ischemic stroke, being of main interest in the etiologic diagnosis of cryptogenic strokes.

Future perspective

Massive sequencing of high-quality, full-length cDNA libraries, coupled with proteomics and functional genomic approaches, will bring a revolution in biomarkers discovery within the next few years. Proteomics seems to be a promising tool for massive biomarker identification in the stroke field, mainly directed at diagnosis and treatment response. A proteomic alternative approach using selected patients with a very clear etiologic profile might provide new promising candidates for each stroke etiology subtype. In addition, recent transcriptomic and GWA studies have incorporated new candidate genes that might generate diagnostic and therapeutic targets for specific stroke etiologies. All generated candidates should be carefully validated before they are implemented in the clinical routine.

The ideal biomarker should be very sensitive, specific, reliable, accessible, standardized, cost effective and easy to interpret. Owing to the complex pathophysiology of the stroke, a combined biomarker panel seems more feasible than a single biomarker for a diagnostic test [101]. Including biomarkers from each stroke etiology or combining biochemical markers with bioimaging markers might be an alternative approach [64].

Although point-of-care tools seem ideal in the stroke unit setting, information would be useful even if obtained during the first days after stroke onset, allowing the use of standard platforms to obtain etiological biomarkers results. The use of such biomarkers would allow a drastic reduction

in the number of undetermined strokes. The identification of CE strokes in cases of paroxistic arrhythmias is one of the main indications since this might produce an intensification of the secondary prevention and might be easily missed in routine examinations. For therapeutical decisions we need a very specific test and for guiding other diagnostic tests a very sensitive biomarker would be needed.

Models of clinical data plus biomarker information and algorithms that are easy to interpret for clinicians would be mandatory if these markers were to be applied in daily practice. It is likely that future well-designed diagnostic trials will lead to clinical validation of selected stroke biomarkers.

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Executive summary

Stroke: 2010 situation

- Stroke is the third leading cause of death and the most common cause of permanent disability in adults worldwide.
- Stroke etiologic classification is critical to discern the best treatment for secondary prevention.
- The etiology of ischemic stroke currently remains undetermined in 25–39% of patients.

Stroke classification systems

- The TOAST classification, which is still the most popular method, is being improved with new approaches to stroke subtyping such as the A-S-C-O (phenotypic) classification or the web-based Causative Classification System (CCS).
- Large-artery atherosclerosis (LAA), cardioembolism (CE) and small vessel disease are the main ischemic stroke subtypes for which an etiologic 'biological signature' might be studied in accessible body fluids.

Stroke biomarkers overview

- The use of biomarkers for etiologic diagnostic assessment might contribute to the reduction of percentages of cryptogenic or undetermined strokes.
- Although there are many biomarkers that have been associated with stroke diagnosis, etiology or prognosis, information regarding clinical utility is extremely limited by the scarcity of large, prospective, rigorous, randomized clinical trials.

Biomarkers to identify stroke etiologies

- Natriuretic peptides (B-type natriuretic peptide) and D-dimer are independent predictors of CE stroke.
- The profile of proinflammatory cytokines (i.e., IL-1β, IL-6 and TNF-α) is elevated in CE strokes as compared with other etiologies. This paradoxical association between CE stroke and inflammation as compared with LAA is now suggested by several studies.
- Haptoglobin-related protein, serum amyloid A, 15-deoxy-δ prostaglandin J2 or Lp-PLA2 have been associated with LAA.
- Some biomarker candidates involved in diseases considered as risk factors for stroke or endophenotypes, such as atrial fibrillation for CE stroke or intima-media thickness and metabolic syndrome for LAA stroke are also promising.
- First studies on stroke etiology and gene-expression arrays and miRNAs have recently been published and genome-wide analyses have identified two new genes related to CE stroke (*PITX2* and *ZFHX3*).

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