Blood biomarkers in ischemic stroke: potential role and challenges in clinical practice and research

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Blood biomarkers in ischemic stroke: potential role and challenges in clinical practice and research

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\section*{ABSTRACT}
Fast and accurate diagnosis of stroke is crucial for the immediate application of the right therapy to patients. However, rapid diagnosis is still a challenge since an ischemic stroke cannot be identified based only on clinical assessment. CT or MRI imaging is required to rule out hemorrhagic stroke since thrombolytic therapy can lead to increased intracranial bleeding and further aggravation of hemorrhagic stroke. In addition, clinical situations that imitate the signs and symptoms of stroke may also impede the rapid diagnosis and treatment of stroke victims. It is therefore of value to discover non-invasive tests that aim to quickly distinguish stroke from stroke mimics and distinguish ischemic from hemorrhagic stroke. Identifying blood biomarkers of stroke is an active area of research since their potential use is not limited to diagnosis and differentiation, but can be applied to prognosis and patient monitoring – monitoring the effectiveness of applied therapy and/or diagnose possible complications. However, their use has been limited so far not only for reasons related to patients and the disease (heterogeneity of stroke etiology, the complexity of the ischemic cascade, the impact of the blood–brain barrier (BBB) on diffusion of blood biomarkers, and difficulties in obtaining consent from stroke patients) but also for reasons that related to laboratory measurement of these biomarkers (pre-analytical and analytical issues as well as interpretation of laboratory measurements). Until today, many biomarkers have been identified, however none so far have shown sufficient sensitivity and specificity in order to be used in the clinical setting. In this review, we will focus on ischemic stroke and we aim to highlight these problems and also investigate if these are due to stroke complexity or due to our limited knowledge of pre-analytical requirements for many of these molecules and the questionable quality of the assays used.

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\section*{Introduction}
Stroke is one of the most important causes of disability and mortality worldwide and is the cause of enormous direct and indirect costs to healthcare services. In the US alone, it is the fifth leading cause of death with in-hospital mortality rates ranging between 11\% and 15\% \cite{1,2}. Recent health statistics show that stroke accounts for 1 out of every 20 deaths in the US \cite{3}. One out of every four stroke victims will experience a recurrent stroke within the next 5 years, approximately half of stroke survivors remain disabled, and 20\% require institutionalization.

Stroke is a complex clinical entity and its identification and diagnosis are based initially on clinical examination of the patient. The two main stroke subtypes are an ischemic stroke (IS) and hemorrhagic stroke (HS). IS represents 85\% of all cases. HS is sub-classified into intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH) (Figure 1). Moreover, these two broad clinical groups are not homogenous.

Defining and categorizing stroke according to pathological process is very important and serves different purposes. In daily practice, it can be used to classify patients for therapeutic decision-making, in research studies it can be used to describe patients' characteristics, and in genetic studies to group patients in epidemiological studies or to phenotype patients \cite{4}.

The system that is mostly used by the clinicians to categorize ischemic stroke has been developed in the multicenter study known by the short name TOAST (Trial of ORG10172 in Acute Stroke Treatment). According to this, IS is divided into the following five major subtypes that underlie the etiology (Figure 1). This is not the
An excellent review examined the pros and cons of each of these systems [4]. Most of these classification systems were developed for specific research projects and their main disadvantage is that they require complete diagnostic investigations for the detection of stroke etiology which is not always feasible in everyday clinical practice.

Ischemic stroke is primarily caused by intracranial thrombosis or extracranial embolism. Atherothrombotic stroke maybe attributed to intracranial or extracranial vessel disease, regarding arteries of the circle of Willis, carotids, and vertebral arteries, as well as aortic arch atherosclerotic disease. Large vessel atherosclerosis (or large vessel disease) accounts for approximately 30% of all IS and is considered to be the cause of a stroke when the reduction of the lumen of the vessel affected is >50% in a cerebral artery. However, a reduction of <50% can cause stroke of this subtype if accompanied by an unstable plaque with ulcerations. Things can become diagnostically uncertain if the patient with a second cause for stroke apart from the large vessel disease presents.

Cardioembolic stroke accounts for approximately 20–30% of all IS and is usually more disabling than non-embolic mechanisms of stroke given the propensity for large intracranial vessel occlusion resulting in larger areas of the ischemic brain. It can result from atrial fibrillation (AF), ventricular thrombus, structural heart defects, aortic arch atheroma, acute myocardial infarction, or valvular heart disease [10–12].

The third subtype, small vessel disease provoking lacunar stroke, is considered to be the cause of stroke when the infarct volume is less than 15–20 mm in diameter and occurs mostly in regions of the penetrating cerebral arteries. This category represents also 20% of total IS. However, infarcts >20 mm in diameter may occur in the same region and with the current diagnostic techniques it remains difficult to determine if the stroke is due to small vessel disease or to other pathophysiology [13].

Strokes of other determined etiology include patients with rare causes of stroke, such as non-atherosclerotic vasculopathies, hypercoagulable states, or hematologic disorders, and represents only a 5% of total IS cases.

Strokes of undetermined etiology represent 20% of total IS cases, including patients where the cause of a stroke cannot be determined with any degree of certainty. The patient either has a negative or incomplete evaluation or the patient presents with two or more potential causes of stroke.

On the other hand, hemorrhagic stroke (HS) can be distinguished as intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH). ICH occurs when a blood vessel within the brain parenchyma ruptures and is characterized by the formation of a localized hematoma within the brain parenchyma and accounts for ~15% of all strokes. The most common cause is hypertension that provokes the rupture of a weak artery. Other causes are arteriovenous malformations, cavernous angiomas, amyloid angiopathy, and tumor metastases. SAH accounts for about 5% of all strokes. Most SAHs are caused by rupture of saccular aneurysms within the subarachnoid space. The terms HS and ICH are used interchangeably as the majority of HS are ICH (12% of the total 20% of HS). Although less common than IS, ICHs are associated with higher mortality [14–17].

It is extremely important to distinguish between stroke subtypes as well as from other clinical situations that “mimic” stroke in clinical presentation, such as migraine, epilepsy, structural brain lesions, etc., all of which can account for up to one-third of the patients that present with “stroke-like symptoms” at the emergency department. Early and accurate diagnosis will lead to prompt and rapid therapy with the existing...
approved drugs. Research for new drugs that could be used in the acute phase is necessary and pharmaceutical industries are actively involved in that process. These therapies aim to protect and/or reverse the rapidly developing necrotic region within the brain.

**Pathophysiology of ischemic stroke**

The pathophysiology of ischemic stroke is extremely complex and involves several processes [18–22]. In this review, we will analyze the mechanisms that underlie ischemic cerebral damage. Understanding these mechanisms will enable us to identify the pathways and the molecules involved that can be potential biomarkers for the early diagnosis and differentiation from ICH and stroke mimicking situations. They may also help to follow up the evolution of an IS and possibly monitor the therapy and predict the outcome.

**Cerebral blood flow is seriously affected during an ischemic stroke**

Ischemic cerebrovascular disease is mainly caused by thrombosis, embolism, and focal hypoperfusion, all of which lead to cerebral blood flow (CBF) disorder. What determines the degree of neurological dysfunction, is the duration of CBF disorder as well as the time of occlusion of the affected vessel, since a decrease in regional CBF leads to diminished tissue perfusion [23–25].

The homeostatic balance in the human brain is regulated by two dynamic mechanisms called cerebral autoregulation (CA) and neurovascular coupling (NVC). In the normal brain, CA is a mechanism responsible for maintaining CBF relatively constant over a wide range of blood pressure (BP) variations. NVC refers to a cascade of events triggered by an increase in neuronal activity leading to an increase in CBF. Both mechanisms are challenged after acute ischemic stroke [26–28]. At rest, the brain receives approximately 20% of cardiac output and it is very sensitive to ischemia. Even very small periods of ischemia on neurons can trigger a complex sequence of events that may result in permanent cerebral damage. The average CBF in an adult is approximately between 40 and 60 ml/100 g/min [24].

During brain ischemia CBF is reduced below a critical level, causing inadequate delivery of oxygen and glucose, and triggering the pathophysiological events that follow acute stroke. This critical event provokes a series of functional, biochemical, and structural changes that lead to irreversible neuronal death. Moreover, the inadequate CBF supply may produce damage to neurons or glial cells beyond that caused by the initial ischemia due to either hypo- or hyper-perfusion [29].

In acute stroke, the tissue that is supplied by the occluded artery is divided into areas irreversibly damaged and into areas that are hypoperfused and therefore can be saved if perfusion is restored timely. This is the base of the meanings of ischemic core and ischemic penumbra [30].

The ischemic core represents tissue that is irreversibly damaged and corresponds to CBF values below 7 ml/100 g/min, whereas the ischemic penumbra represents tissue that is functionally impaired but structurally intact and potentially salvageable. This area corresponds to CBF between 17–22 ml/100 g/min (high limit) and 7–12 ml/100 g/min (low limit). Another compartment in the brain is defined as oligemia and represents mildly hypoperfused tissue with CBF between 22 and 40 ml/100 g/min [31]. This area of oligemia is not at immediate risk of infarction but under certain circumstances (hypotension, fever, acidosis etc.) can be easily incorporated into penumbra and subsequently undergo infarction (Figure 2) [20,32].

As blood flow declines, the functional neuronal activity is affected first, and as the ischemia progresses, the metabolic activity required to maintain the structural integrity of the cells gets suppressed [33]. This leads to the activation of several pathways that may be neuroprotective or detrimental to the brain. These events occur in an overlapping manner and depend on the intensity and duration of ischemic insult. These are (Figure 3):

1. energy failure,
2. excitotoxicity,
3. energy failure,
4. excitotoxicity,
3. oxidative stress,
4. disruption of the blood–brain barrier (BBB),
5. inflammation,
6. hemostatic activation, and
7. cell death (necrosis or apoptosis).

Energy failure

The energy needs of the brain in oxygen and glucose are relative high, and almost exclusively depends on oxidative phosphorylation. The reduction of regional blood flow results in insufficient delivery of oxygen and glucose to neurons and causes brain damage by activating an ischemic cascade which restricts the brain from other essential substrates. As a consequence, brain cells fail to produce high-energy phosphate compounds (ATP). This procedure rapidly leads to the dysfunction of all energy-dependent ion transport pumps of neurons and glia [34]. Loss of ion pump function leads to loss of potassium and accumulation of sodium, chloride, and calcium. This is accompanied by an influx of water that results in rapid swelling of neurons and glia (cytotoxic edema). The extent of damage depends on duration, severity, and location of the ischemia. Moreover, reduced oxygen availability results in anaerobic glycolysis and accumulation of lactate. High lactate levels are considered as a marker of anaerobic metabolism in stroke and a possible cause of secondary damage that may lead to infarct expansion and poor outcome [35].

Excitotoxicity

The loss of ion pump function also leads to an uncontrolled depolarization of neurons in the penumbra. This results not only in intracellular accumulation of sodium and calcium ions but also in excessive release of glutamate, which is an excitatory neurotransmitter in the perisynaptic terminal. Simultaneously the re-uptake of glutamate from the extracellular space is reduced. The presence of excessive amounts of excitatory amino acids in the synapses and extrasynaptic sites can eventually lead to neuronal death in a process known as excitotoxicity (Figure 4). Although excitotoxicity usually refers to the injury and death of neurons, other cells such as astrocytes may also be damaged as a result of the increased levels of glutamate. The excitatory effects of glutamate are mediated through two kinds of receptors: the ionotropic receptors and the metabotropic receptors. These are located in the pre- and post-synaptic neuron membranes of the central nervous system. Practically all glutamate receptors are involved in the excitotoxic process. However, the N-methyl-D-aspartate receptor (NMDAR) is considered the key initiator of excitotoxic damage. The glutamate overload leads to prolonged stimulation of ionotropic receptor subtypes (AMPA and NMDA), which enhance the excessive influx of calcium, sodium, and water into neurons. Massive calcium influx activates catabolic processes mediated by various proteases, lipases, phosphatases, and endonucleases. This results in the activation of several signaling pathways, mainly causing overproduction of free radicals, mitochondrial damage, cell membrane disruption, and DNA fragmentation, which damage the cell structure and synergistically result in neuronal death [21,36,37].

Oxidative stress

Oxidative stress (OS) constitutes a uniform mechanism of injury of many types of disease processes. It occurs when there is an imbalance between the production of free radicals from reactive oxygen species (ROS), reactive nitrogen species (RNS), and the endogenous scavenging capacity of cellular antioxidants in the body [38,39].

ROS and RNS are families of highly reactive species and important mediators of cell growth, adhesion, differentiation, and apoptosis. However, when produced in excess they cause cell damage either directly or as intermediates in diverse signaling pathways [40]. The ROS which are particularly responsible in oxidative stress include superoxide radical anion (O$_2^-$), hydroxyl radical (OH$^-$), hydrogen peroxide (H$_2$O$_2$), nitric oxide (NO*), and peroxynitrite among others. Particularly NO* and O$_2^-$ are two free radicals that react with each other to form the powerful oxidant peroxynitrite (ONOO$^-$) [41].

Antioxidant mechanisms are present in cells to facilitate ROS removal or to prevent their generation. Major antioxidants are enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), as well as non-enzymes like vitamin E, vitamin C, and reduced glutathione (GSH). During OS, the rapid overproduction of free radicals overcomes their capacities in detoxification and scavenging capacities.

The brain is particularly vulnerable to ROS and RNS increases due to low neuron antioxidant enzyme activity and high concentrations of peroxidizable lipids, high oxygen consumption, and high levels of iron, all acting as pro-oxidant under pathological conditions. ROS production is considered as an important mechanism of brain injury after exposure to ischemia-reperfusion [38,42]. Oxidative stress is a significant injury during brain ischemia since these free radicals attack cellular proteins, DNA, and lipids. OS leads to inflammation, an increase in the permeability of the BBB, edema formation, and activation of apoptotic, necrotic autophagy pathways. All of these are determinants of the final infarct size [38].
Figure 3. Graphic representation of the main pathophysiological mechanisms that lead to permanent cerebral damage in acute ischemic stroke.
Antioxidant supplementation has been considered in clinical trials as a means to improve outcome not only in patients that cannot be considered for thrombolysis but also in patients who are undergoing this treatment and are at risk of the so-called ischemia-reperfusion injury [38].

**Inflammation**

There is increasing evidence that inflammation plays a role in both the development and the progression of stroke [43–46]. The inflammatory response is mediated by pro-inflammatory prostaglandins, cytokines, and chemokines. These factors stimulate immune cells which activate the adaptive immune system leading to the release of acute phase reactants. Some of these pro-inflammatory proteins [IL-6, IL-1β, complement proteins, C-reactive protein (CRP), serum amyloid A (SAA), coagulation proteins, and fibrinogen] act on endothelial cells. The acute effect of this action is the upregulation of thrombotic mechanisms while the long-term effect is the contribution to the formation and maturation of atherosclerotic plaques. Therefore, inflammation plays an important role to the pathogenesis and progression of atherosclerosis, plaque rupture, platelet aggregation, intravascular thrombosis, all of which are involved in the pathogenesis of stroke (Figure 5).

The association between acute inflammatory response and the onset of stroke can be seen in various states of acute infection. Several studies have suggested that recent infection increases the risk of stroke. However, the exact relationship between infection, inflammation, and cerebrovascular events is not fully elucidated. Chronic infections have also been associated with cardiovascular events and ischemic stroke. The link is believed to be the result of chronic low-level inflammation (Figure 4). However, a causative role of chronic infection in CVD and stroke has not been established yet since all data come from epidemiological studies, and there are no results from clinical trials involving antibiotic treatments [45–47].

The interactions between the brain and the immune system (innate and adaptive) are related to tissue damage, systemic infection, and tissue regeneration after stroke. The following are several areas in which inflammation seems to be involved in acute stroke.

1. As an indicator of underlying vascular disease that predisposes the patient to stroke.
2. As the process that acts as a trigger for an acute event.
3. Once the stroke has occurred, as the acute response to tissue injury (with possible roles in the exacerbation of the injury or its consequences).
4. As a prognostic indicator in the acute phase.
5. As a prognostic indicator for recurrent cerebrovascular events.

Any or all of the above may be potential therapeutic targets. The inflammatory mechanisms have been examined in detail in several reviews [48–52]. Focal ischemia causes hypoxia and interruption of glucose supply, triggering a strong inflammatory response within the first

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*Figure 4. Excitotoxicity. In neurons, due to reduced blood flow, the lack of oxygen interrupts oxidative phosphorylation and reduces cellular ATP production. ATPase pump is inhibited and leads to loss of cellular membrane ionic gradient, increased influx of extracellular Ca++ and increased the release of glutamate (modified from reference [395]).*
few hours of the onset of stroke, resulting in a massive cell necrosis within the infarct core. These dying cells release signals known as DAMPs (damage-associated molecular patterns) which are endogenous molecules that induce the activation of toll-like receptors (TLRs). TLRs are the receptors for advanced glycation end products (RAGE) and others that amplify the expression of inflammatory mediators and amplify tissue damage [53,54]. These DAMPs are collectively called alarmins because they induce chemotaxis and interact with receptors on antigen presenting cells, and they link innate immune activation to the activation of the adaptive immune response.

These signals activate microglia, the resident macrophages in the brain, and peripheral leukocytes, which release pro-inflammatory cytokines that upregulate the expression of adhesion molecules and stimulate the synthesis of chemokines. Increasing evidence also shows that astrocytes may also act as inflammatory cells responding to ischemic stroke [50]. Simultaneously, an increase in BBB permeability allows leukocyte infiltration into the brain in order to allow the clearance of the debris caused by the cell death [55,56]. This infiltration in combination with the activation of microglia has both beneficial and detrimental effects on stroke outcome if it persists over time. Furthermore, the permeability leads to an excess of inflammatory cytotoxic mediators that prolong the inflammatory response, increase the brain damage and contribute to secondary complications such as edema or hemorrhagic transformation [49,57].

Some potential DAMP signals that have been identified to date include the chromatin-associated protein, high mobility group box-1 (HMGB1), heat shock proteins (HSP), purine metabolites such as adenosine triphosphate (ATP) and uric acid, S100 proteins, DNA, and RNA [58–60].

One of the most well-studied DAMPs in ischemic stroke, HMGB1, is a highly conserved non-histone nuclear DNA-binding protein, expressed in most eukaryotic cells including neurons. In healthy cells, HMGB1 stays inside the nucleus, where it interacts with DNA and histones to determine chromatin structure and regulate key processes such as transcription. HMGB1 has other intracellular actions since, when translocated to the cytoplasm, it can mediate autophagy. However, cells undergoing necrosis-death immediately release HMGB1 to extracellular space. Outside the cell, HMGB1 acts as a DAMP. It interacts with pathogen-associated molecular patterns (PAMPs), cytokines and chemokines. As such, HMGB1 can play multiple roles in the pathogenesis of the inflammatory and autoimmune disease and mediate processes that range...
from inflammation to repair. It is also a pro-inflammatory factor capable of stimulating the production of other inflammatory factors and plays an important role in sepsis and cerebral ischemia-caused inflammatory injury [61–64].

**Stroke-induced immune-depression and post-stroke infections**

Post-ischemic inflammation is a self-limiting process that eventually subsides and prepares the structural and functional reorganization of the injured brain. The factors that govern the resolution of inflammation and the reestablishment of tissue homeostasis in the brain are still poorly understood. Increasing evidence suggests that this process is not passive due to exhaustion of the inflammatory signals but is orchestrated by the interplay of a large number of mediators actively suppressing the inflammatory response [65–67]. The major steps to resolve the inflammation is the removal of the dead cells by phagocytosis (by microglia and macrophages) and the production of immunomodulatory cytokines, such as transforming growth factor (TGF-β) and interleukin 10 (IL-10). The first helps to suppress inflammation and the second have neuroprotective and anti-inflammatory properties and its release help the resolution of inflammation [68]. However, in patients with severe stroke, there is a positive correlation between the extension of brain lesions and the strength of the neuroinflammatory reaction [60]. In extreme cases, the massive overexpression of circulating pro-inflammatory cytokines seem to be the major cause of the immune system’s inability to respond to secondary stimuli elsewhere [69]. Such acute phase response is followed by a dramatic immunodepression, characterized by lymphopenia, reduced functional activity of monocytes, upregulation of anti-inflammatory cytokines, lymphocyte apoptosis, and splenic atrophy. These immunological changes are associated with an increased tendency for respiratory and urinary tract infections, responsible for significant morbidity, and mortality in stroke patients [68].

It is not completely understood how these systemic immune changes are mediated but one hypothesis and increasing evidence suggests that the over-activation of peripheral immune cells may lead to exhaustion of mature leukocytes (mature monocytes) causes an increase in myeloid production and recruitment of immature leukocytes. This subpopulation is unable to respond appropriately to various insults resulting in deregulation of the immunological signaling pathways and ultimately to immunodepression [53,69,70]. Moreover, this “sterile” inflammatory response leads to overproduction of pro-inflammatory mediators (from systemic innate immune cells) hours after the brain lesion.

Post-stroke immunodepression seems to be mediated by catecholamines and steroids released by the SNS. IS gives rise to an intense activation of the SNS and the release of catecholamines. Norepinephrine (the primary SNS neurotransmitter) is released into the lymphoid tissue and modulates the function of immune cells. The stroke-induced SNS activation is responsible for lymphopenia, impaired function of monocytes, a shift from Th1 to Th2 cytokine production, and increased lymphocyte apoptosis in the affected patients [44,71–73]. The result is inhibition of TNF-α, IL-1, IL-12, IFN-γ, and nitric oxide (NO) production and increased production of IL-6 and IL-10 by the immune cells [44,74]. Moreover, clinical studies showed that catecholamines have an immunosuppressive effect after stroke [71]. Increased activity of the SNS after stroke is associated with a higher risk of infection [75].

Elevation of pro-inflammatory cytokines and several chemokines after brain ischemia can increase production of corticotropin-releasing factor by stimulation of certain neurons in the hypothalamus. Furthermore, blood-borne cytokines may augment cortisol secretion by enhancing adrenal function [76,77]. Studies have shown that in patients with acute stroke, there is a correlation between increased IL-6 concentrations in the plasma and CSF and elevated levels of plasma adrenocorticotropic hormone and cortisol [77,78]. Finally, glucocorticoids suppress the production of pro-inflammatory mediators, promote the release of anti-inflammatory cytokines (i.e. IL-4, IL-10), and TGF-β [79,80].

The activation of these mechanisms immediately after an acute ischemic cerebrovascular event, contribute to stroke-induced immunodepression syndrome (SIDS), increased risk of recurrent stroke and death, and can account for approximately 30% of the mortality in stroke patients [81–87]. Depending upon classification, it has been reported that the incidence of post-stroke infections is between 23 and 65%, with pneumonia and urinary tract infections (UTI) being the most common [88–90]. The true assessment of infection after stroke is challenging since standardized criteria for diagnosis are lacking [90]. A recent meta-analysis of 87 studies involving 137,817 patients found that infection rates after stroke vary considerably, ranging from 5% to 65%. Differences in patient populations, study design, and the definition of infection across studies may account for these large variations in post-stroke infection rates [81]. Biomarkers may facilitate in early diagnosis of infections in patients with acute stroke and to monitor the efficacy of pharmacological therapies applied to these patients.
**Hemostatic activation**

Thrombotic and inflammatory processes are two highly interconnected factors that lead to cerebral vessel occlusion and ultimately to stroke. Endothelial stress is involved in the early pathophysiology of stroke. At the sites of vascular injury, the sub-endothelial extracellular matrix (ECM) is exposed to the blood, which triggers adhesion and activation of blood platelets (primary hemostasis), followed by the activation of the coagulation system (secondary hemostasis). The aim is to form a fibrin-rich thrombus that seals the wound and initiates wound healing [91].

Under physiological conditions, hemostatic balance is maintained through the complex interplay of pro-coagulant, anti-coagulant, and fibrinolytic factors. Key constituents of this the hemostatic system are von Willebrand factor (vWF) and platelets. Platelets, under normal conditions, circulate in close proximity to the endothelium without forming stable adhesion contacts due to the anti-adhesive properties of inactive endothelial cells. However, after vascular injury, platelets rapidly adhere to sites of endothelial injury to establish a hemostatic plug that prevents excessive blood loss. This process is critically dependent on the efficiency of platelet adhesion to the subendothelial matrix, as well as on the ability of the platelets to undergo rapid biochemical and morphological changes that support aggregation and the localized activation of the coagulation cascade. vWF is a large, highly adhesive, multimeric glycoprotein that is found mostly in plasma and is produced by endothelial cells and megakaryocytes (precursors of platelets). vWF is critical for hemostasis and thrombus formation for two reasons. First, it acts as a bridge molecule for the adhesion and aggregation of platelets at the sites of vascular injury. Second, it is also a carrier molecule of factor VIII protecting it from rapid clearance and increasing its half-life. Therefore, it is an essential molecule for both primary and secondary hemostasis [92].

vWF is synthesized as a single pre-polypeptide chain and after removal of the signal peptide in the endoplasmic reticulum is dimerized and undergoes further maturation in the Golgi apparatus where it is multimerized. vWF that is produced in endothelial cells is either released directly into the plasma or stored in the Weibel-Palade bodies. Whereas vWF that is produced in megakaryocytes, is stored in platelet a-granules until platelet activation [93,94].

The relative contribution of endothelial or platelet-derived vWF to hemostasis is under investigation. Recent evidence suggests that platelet-derived vWF might aggravate thrombo-inflammatory diseases such as stroke [95,96]. This is probably due to differences in glycosylation, affinities for platelet receptors, and different multimer distribution (platelets preferably express higher molecular weight multimers) [95].

vWF exists in various molecular sizes (vWF multimers) and include low, medium, high, and ultra-large molecular weight forms. In plasma, it circulates at approximately 5–10 μg/mL as multimers with a molecular weight ranging from 500 to 20,000 kDa [97]. Plasma vWF concentrations vary widely even among healthy individuals. The reason for this variability is unclear, but polymorphisms in the gene that encodes vWF seem to be important [98].

The ultra-large multimers are not typically found in plasma because they undergo rapid proteolysis which reduces them into smaller multimers. Of all vWF multimers, the high-molecular-weight is those with the greatest hemostatic activity (the most pro-coagulant forms of vWF), because of their binding capacity for collagen and the platelet receptors glycoprotein Ib and IIb/IIIa and platelet aggregation. Because of this role of vWF, it was logical to assume that a correlation between higher plasma levels of vWF and the development of cardiovascular disease exists. There is a recognized association between vWF and endothelial dysfunction. Circulating vWF has been shown to be increased in subjects with abnormal endothelial function and considered an indicator of vascular endothelial health [99,100]. Experimental studies have shown that animals with reduced levels of vWF are protected from developing aortic plaque even when fed a cholesterol-rich diet. Moreover, in experimental models of acute stroke, animals with vWF deficiency were protected from brain ischemia-reperfusion injury and the infarct sizes were significantly lower from animals with normal vWF activity [96,101].

Several human case–control studies have shown an association between high vWF plasma levels and stroke [102–106]. However, the interpretation of the results of these studies is not easy. First, there is no uniform post-stroke time-point of vWF measurement. The assays that have been used to measure vWF are not uniform making the comparison of the results impossible. Several prospective studies have also identified that high vWF plasma levels are a strong predictor of stroke [107–112].

Another factor, ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin motif repeats 13) a recently discovered metalloprotease, which belongs to the ADAMTS family, appears to be an important enzyme. It cleaves the ultra large vWF multimers into smaller forms (specifically cleaves the bond between tyrosine-842 and methionine-843 in the A2 domain of vWF multimers), counter-acts their pro-thrombotic activities, and has antithrombotic properties. A deficiency of ADAMTS13 is seen in patients with thrombotic thrombocytopenic purpura, which is characterized by
cerebral ischemia caused by platelet thrombosis in the cerebral microcirculation. Therefore, ADAMTS13 is considered a biological regulator of vWF activity [96]. Experimental studies have shown the anti-thrombotic and anti-inflammatory properties of ADAMTS13. Moreover, several human studies have shown that low ADAMTS13 activity is associated with higher risk of ischemic stroke [103,113–119].

Consequently, it seems that pharmacological interference in vWF-mediated platelet adhesion could have a clinical benefit. Indeed, a number of vWF blockers have been studied, however, none of them have yet to achieve regulatory approval for marketing. Results from preclinical animal and clinical patient studies demonstrate that inhibition of these interactions holds great promise as an antithrombotic strategy without significant increase of the bleeding risk [96,120]. Apart from blocking binding of vWF to either collagen or GP-Iba, another way of reducing vWF activity is to reduce its size by ADAMTS13. Recombinant ADAMTS13 has been developed as a new therapeutic agent. Although it was found to be protective, it is still in pre-clinical studies and more data are needed before it proceeds into human clinical trials [115,121]. Moreover, in order for these agents to be introduced into clinical trials involving humans, standardized laboratory assays need to be established for the measurement of vWF and ADAMTS13.

Blood–brain relationship: not so simple

The BBB, a dynamic interface between the peripheral circulation and the central nervous system (CNS), and its integrity are important for the maintenance of the homeostasis of the CNS. The BBB has three main functions [122–125]:

i. it strictly limits the passive diffusion of macromolecules and pathogens from the blood to the brain,
ii. it mediates the transport of nutrients to the brain parenchyma as well as the efflux from the brain of toxic metabolites and xenobiotics, and
iii. it regulates the migration of circulating immune cells.

The BBB is composed primarily of endothelial cells linked by tight junctions that largely prevent communication at the molecular level between plasma and the central nervous system. Only capillary vessels have complete BBB properties [126]. As the diameter of blood vessels increase, their permeability also increases [126–128]. The BBB is tightly controlled by pericytes, embedded in the vascular basement membrane, microglial cells and perivascular macrophages, astrocytes, and neurons which altogether constitute the neurovascular unit (NVU), a concept highlighting the functional cell–cell interactions supporting BBB function [129,130]. Recently, physiological studies of cerebral blood flow dynamics have demonstrated that substantial intercellular communication occurs between cells of the vasculature, the neurons, and the glia. These findings suggest that the BBB does not function independently, but as a module within the NVU. Cell-cell interactions in the NVU form the basis for brain function. Dysfunctional signaling in the NVU underlies the basis of disease. Alterations in micro-vessel integrity may have other effects within the NVU that affect neuronal function. The mechanisms of NVU response to stroke are not fully understood. However, any fully effective stroke therapy must include both prevention of cell death as well as repair of integrated neurovascular function [131]. The integrity of BBB plays an important role in the pathophysiology of acute stroke since it protects the neuronal microenvironment. BBB degeneration after acute ischemic stroke (AIS) may lead to pathologic processes such as edema and hemorrhagic transformation (HT) [127,128,132,133]. Several studies have shown that BBB disruption in acute ischemic stroke varies considerably (from 15% to 66%), and it depends on stroke severity, the timing, and the methodology of the evaluation [57].

Several mechanisms contribute to ischemic damage of the BBB, which appears to be biphasic, particularly after reperfusion. In this model, an early increase in permeability is followed by a refractory period during which BBB permeability returns to baseline, and a delayed second increase in permeability [57].

Recent studies, however, have challenged this interpretation by showing incomplete recovery after the first increase in BBB permeability, which indicates a continuous leakage for days to weeks without any appreciable change in magnitude. Longitudinal studies assessing BBB permeability after stroke to date have been limited to animal models and only recently human studies seem to confirm this continuous leakage theory [134,135].

The extent of BBB disruption is associated with the type, severity, and duration of ischemic insults. The molecular mechanisms underlying BBB opening are not fully understood, although several matrix metalloproteinases (MMPs) or matrixins are believed to regulate BBB permeability and function during ischemic stroke [131]. Their role is to cleave protein substrates based on a conserved mechanism that involves the activation of a site-bound water molecule by a Zn^{2+} ion. MMPs consist of 23 distinct proteases in humans (and 24 in mouse). Excreted MMPs are generally classified according to their substrate specificity, which gives four groups of MMPs: the collagenases which contains the MMP-1, -8, and -13, the gelatinases (MMP-2 and -9), the stromelysins (MMP-3, -10, and -11) and a heterogeneous group containing the rest: matrilysin (MMP-7), metallo-elastase...
(MMP-12), enamelysin (MMP-20), endometase (MMP-26), and epilysin (MMP-28) [136]. Most of the MMPs are synthesized as inactive latent enzymes. Conversion to the active enzyme is generally mediated by activator systems that include plasminogen activator or the pro-hormone convertase, furin.

MMP activity is regulated by a group of endogenous proteins called tissue inhibitor of metalloproteinases (TIMPs), which bind to active and alternative sites of activated MMP. Four TIMPs have been identified today [131,137]. Experimental studies have shown that deletion of TIMP1 or TIMP2 coding genes results in overexpression of MMP9 accompanied by exacerbated BBB disruption, neuronal apoptosis, and ischemic injury. Consequently, MMPs could represent potential targets for pharmacological interventions and the inhibition of their activity may have a beneficial effect [138].

The injured brain has various cell types that can express MMPs including resident and infiltrating inflammatory cells. Astrocytes normally secrete MMP2 from their end-feet. Endothelial cells (and particularly microglial cells and pericytes) secrete MMP3 and MMP9, and finally, neutrophils which are the main source of MMP8 [138,139]. The expression of MMPs in the normal adult brain is very low to undetectable, but clinical and experimental studies have shown that several MMPs are upregulated and activated after ischemic stroke. Animal and human studies have shown that MMP-2, normally is present in the brain around the cerebral vessels in the astrocytic end-feet and at the ependymal and pial surfaces. Astrocytes produce latent MMP-2 which becomes activated with the onset of an ischemic insult. This early opening of the BBB can be a therapeutic opportunity blocked by treatment with an MMP inhibitor. However, in the absence of a treatment, the permeability reverts to normal several hours later (reversible opening). After 24–48 h, there is a second, more severe disruption of the BBB. The second opening of the biphasic BBB pattern of injury may last for several days and may be associated with hemorrhage and vasogenic edema (delayed opening). During the secondary phase of vessel injury, the levels of MMP-9 are markedly increased. Induction of MMP-9 is mediated by cytokines and immediate-early genes. Activation of the proforms occurs by the action of other proteases and of free radicals. In cell culture, microglial cells produce an activator of proMMP-9, possibly MMP-3 or free radicals, and less likely plasmin. Pericytes are involved in activating MMP-9. During the secondary phase of BBB disruption, which occurs at 48 h the risk of hemorrhage is increased. Vasogenic edema contributes to the cytotoxic edema already present. Disruption of the BBB by proteases occurs early in inflammation, where the white blood cells use MMPs to enter the brain, setting in motion the release of MMPs by astrocytes and pericytes. In the absence of hypoxia, the cells remain intact, and once the inflammatory response subsides, recovery is possible. During an ischemic/hypoxic insult, the endothelial cells are damaged secondary to the induction of MMPs by endogenous brain cells. There is an increased risk of bleeding, and recovery is impaired due to the extensive hypoxic injury to the cells [57,70,131,138,139].

However, despite their harmful effects MMPs when they are overexpressed in CNS injuries are also known to be key mediators of tissue remodeling during stroke recovery. Recent evidence shows that MMPs participate in two major processes: angiogenesis and neurogenesis. It is known that MMPs increase the availability of growth factors (i.e. vascular endothelial growth factor) involved in tissue remodeling and vascularization by processing their precursors and that they modulate the extracellular matrix to allow the mobilization of both progenitor vascular and neuronal cells and stimulate the formation and growth of new neurons in adjoining regions [140–142].

Therefore, MMPs play a dual role in stroke. During the acute phase, they contribute and increase tissue damage while during the recovery phase they participate in remodeling processes. This dual role is comparable with the duality of inflammation in stroke. MMP inhibition has been suggested as a possible therapeutic intervention to minimize and even prevent BBB disruption during the early phases of ischemic stroke. However, the timing of therapy initiation and termination seems to be critical since experimental studies have shown if treatment with MMP inhibitors is applied late in the course of an acute stroke, neurovascular remodeling is suppressed, ischemic brain injury is increased, and functional recovery is impaired [138,143,144].

Finally, we must note that the disruption of BBB integrity can facilitate the delivery of drugs into the ischemic brain and the release of proteins or their fragments, deriving from lysed brain cells, into the systemic circulation. The latter can be used as possible biomarkers reflecting the pathophysiological processes that take place after an acute stroke.

**Cell death in cerebral ischemia-apoptosis versus necrosis**

At the cellular level, the biochemical and electrophysiological mechanisms involved in the ischemic brain injury vary according to the extent of cerebral ischemia. The characterization of cell death pathways in stroke is crucial for the development of effective therapeutic interventions. Neuronal cell death occurs as a result of two main mechanisms: necrosis and apoptosis. Necrosis is a process not programmed or regulated and is the...
mechanism that follows acute permanent focal vascular occlusion, and occurs mainly as a consequence of disruption of cellular homeostasis due to energy failure. It is usually accompanied by cellular swelling, membrane lysis, inflammation vascular damage, and formation of edema. Apoptosis or programmed cell death is characterized by cell shrinkage, chromatin aggregation with preservation of cell membrane integrity and mitochondria with lack of inflammation, and injury to surrounding tissue.

Although apoptosis and necrosis represent distinct modes of cellular death, they coexist within an injured tissue. Necrosis is the main mechanism of cell death within the necrotic core while apoptosis occurs in the ischemic penumbra where the degree of ischemia is milder and the available energy allows the expression of apoptotic proteins [20,145,146]. Research in the past decade has revealed that during the first few hours after a stroke, neurons in the ischemic penumbra, suffer transiently reversible damage and then ultimately undergo death by apoptosis [147–150]. Therefore, the initial few hours of reversible neuronal injury offers an opportunity for therapeutic intervention. For a detailed review of programmed cell death in cerebral ischemia, the reader is referred to the article of Graham et al. [151].

**Hemorrhagic transformation of ischemic stroke**

Intracerebral hemorrhagic transformation (HT) refers to the extravasation of blood products from vessels after ischemic stroke. It is a serious complication of acute IS, and contributes to increased early mortality affects functional recovery and is worsens acute stroke [152,153]. HT occurs in 10–40% of ischemic stroke cases [154,155], and is especially common in cardioembolic stroke where it is found in 74% of cases within the first 4 days [156,157]. HT can occur spontaneously but its risk is especially high when patients receive thrombolytic reperfusion therapy with tissue plasminogen activator [158]. The frequency and definition of HT varies among clinical studies, making comparisons difficult [159]. A more objective radiologic definition for ICH has been proposed: “hemorrhagic infarction, described as petechial hemorrhages within a hypodensity, and parenchymal hematomas (PHs) were hemorrhages extending beyond the hypodensity with varying amounts of mass effect” [159]. The clinical spectrum of HT also varies and is usually graded according to ECASS II in four subtypes [160]. All of them are associated with poor outcome [160,161].

Even the so-called asymptomatic HT can worsen outcome several weeks or months after the onset of stroke [162,163]. Reperfusion therapy contributes to better clinical outcome in acute ischemic stroke but carries the risk of a significant IHT compared to supportive care [158,164–166]. Identifying ischemic stroke patients at risk of IHT could have substantial clinical implications, especially in the setting of thrombolytic therapy. Acute stroke patients at very high risk for IHT could foreseeably have alternative treatment, such as a modified dose of thrombolytic agent or co-administration of a second agent able to reduce IHT risk [167,168].

**Diagnostic approach:** At present the only “weapon” clinicians have to identify those at risk to develop HT is neuroimaging and clinical evaluation [159,169,170]. However, neuroimaging techniques are only available in few stroke centers. In addition, the presence of contraindications may limit the use of neuroimaging in many patients. Therefore, it would be very helpful to find additional approaches to evaluate and follow up patients at risk for HT. Biochemical markers seem to play an important role in this direction.

**Mechanism of post-stroke hemorrhagic transformation:** Intracerebral hemorrhagic transformation is a complex phenomenon that frequently accompanies ischemic stroke, mainly because of disruptions of the blood–brain barrier (BBB) [171–173]. Post-stroke HT occurs when BBB permeability increases. Immediately after ischemia onset, ATP decreases, causing a subsequent loss of Na+/K+ ATPase activity. Further rupture of BBB damages the whole neurovascular unit, which consists of the extracellular matrix, endothelial cells, astrocytes, neurons, and pericytes [174]. Therefore, neurovascular injury can significantly extend parenchymal damage into irreversible infarction and pan-necrosis [175].

**Role of biomarkers in stroke research and clinical practice**

**What is a biomarker?**

Biomarkers are defined physiological characteristics or biological substances that can be measured and monitored objectively and reproducibly. They can be used as indicators of physiologic or pathological processes, risk factors, or pharmacological responses to therapeutic interventions. Sensitivity refers to the ability of a biomarker to detect the presence of a disease when the disease is present and specificity refers to its ability to exclude the disease when it is not present [176,177].

**Where biomarkers can be used?**

In order for biomarkers to be useful in stroke, they need to provide answers to several clinical, epidemiological, and research situations [178–182]:

1. Predict the risk of stroke,
2. Clarify pathological mechanisms,
3. Early and accurate diagnosis of stroke and its subtypes in the acute setting, (differentiate stroke from mimics, and ischemia from hemorrhage),
4. Follow-up patients in order to monitor stroke evolution,
5. Predict outcome,
6. Predict and monitor complications,
7. Selection and guidance of therapy, and
8. Clinical research and drug development.

Given the complexity and heterogeneity of stroke pathophysiology and the diversity of clinical and research questions that must be answered, it is obvious that a single biomarker cannot give us all of the answers we seek [183].

How to select biomarkers – biomarker discovery techniques

In the acute setting and depending on the clinical question to be answered, there is a need for markers that either are upregulated in the very early phase (if the aim is diagnosis of stroke subtypes and differentiation from mimics or ischemia from hemorrhage) or upregulated and peak later, providing information on outcome and can be used to guide therapeutic decisions.

There are different approaches in the selection of candidate molecules for biomarkers. The most obvious is to focus on the specific pathways that are known to be involved in the cascade of the pathophysiological events of ischemic or hemorrhagic stroke and find biomarkers that reflect:

1. Increased oxidative stress,
2. Glial and/or neuronal death,
3. BBB disruption,
4. Inflammation,
5. Hemostasis, and

Using this approach, a large number of potential biomarkers have been investigated by several research groups. In this review, we focus on this approach and in Table 1, we summarize biomarkers that have been more intensively investigated and that either already exists in automated in automated platforms or show promise for future automation.

Another approach is to use global profiling methods (the –omic approach) such as gene expression, proteomics, and metabolomics. These methods permit the large-scale analysis of the expression products of genes in patients with stroke. The biological samples that are available for such analysis are usually blood samples since brain tissue is not available for these studies. The reader is referred to several excellent reviews that analyze these methods and the newly developed techniques that have been used for their discovery [184–198].

General considerations concerning biomarker use

The evolution from promising discoveries in biomarker research to tests that are used in routine clinical setting is complex. The greatest challenge is the lack of a true reference standard as diagnostic or prognostic tool for stroke. This is because of the heterogeneity of stroke, the complexity of its pathophysiology, and the presence of BBB. Therefore the “gold standard” for the diagnosis and prognosis remains the clinical decision by an expert clinician.

Vasan [177] has defined a number of characteristics that a new biomarker must have in order to be of clinical value (regardless of the purpose for its use):

- it is accurate and reproducible,
- it is obtained in a standardized fashion,
- it is acceptable to the patient,
- it is easy to interpret by clinicians,
- has high sensitivity and specificity for the outcome it is expected to identify,
- it explains a reasonable proportion of the outcome independent of established predictors consistently in multiple studies, and
- there are data to suggest that knowledge of biomarker levels changes management.

Almost all biomarkers that have been used in research studies so far have been selected either because they are associated with known pathophysiological mechanisms of stroke, or empirically by screening postmortem CSF or performing proteomic analysis [199,200]. The human proteome project an international scientific collaboration (https://www.hupo.org/human-brain-proteome-project) might help to identify potential biomarkers with proteomic techniques [201].

At present, blood biomarkers have performed poorly. In case–control studies where stroke cases were compared with controls, there were significant differences in the mean values of several biomarkers. However, distribution of values show significant overlap and when these biomarkers are tested in prospective studies show limited value. There are several reasons that can explain this bad performance some of them related to assays themselves and some to stroke as a clinical entity.
### Table 1. Potential blood biomarkers in the pathophysiological processes of stroke.

<table>
<thead>
<tr>
<th>Biomarker groups associated with glial cells</th>
<th>Biomarker</th>
<th>Description</th>
<th>Potential role in stroke management</th>
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<tr>
<td>Protein biomarkers associated with glial cells</td>
<td>s100β</td>
<td>Function: a calcium-binding protein involved in cell cycle progression and differentiation; Cell–cell communication (astrocyte-neuron); cell growth; intracellular signal transduction; involvement in development and maintenance of the CNS. Localization: Astrocytes; Schwann cells; Melanocytes; Adipocytes</td>
<td>May predict risk for hemorrhagic transformation after thrombolytic therapy. Could be used in stroke prognosis and prediction of infarct or hematoma volume. Correlates with severity, and functional outcome. Several automated and manual assays are available but comparison studies have shown poor agreement and large biases preventing the interchangeable use of results. Lack of standardization matrix effects and differences in antibodies used may explain these differences. Long term storage may affect sample integrity. Very limited data on biological variation. Use of anticoagulants for sample collection may increase results.</td>
<td>[259–265] [266–270]</td>
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| GFAP (glial fibrillary acidic protein) | | Function: Major constituent of intermediate filament protein. role in cell structure, BBB, communication. Localization: astrocytes | Differentiation between IS and HS. Several in-house and commercial research (ELISA) assays (automated and multiplexed) have been used in studies but results may differ due to lack of standardization (lack of comparison studies). Knowledge of pre-analytical variables that may affect results is very limited. | [271–274] |

| Protein biomarkers associated with neuronal cells | NSE (neuron specific enolase) | Function: Dimeric intracellular neuronal glycolytic enzyme. Localization: Neurons; Cells of the diffuse neuroendocrine system; Erythrocytes | Prediction of infarct volume. Several automated and manual assays available but results differ due to differences to antibodies used in assays and lack of a common standard. Long-term storage does not affect levels. Hemolysis should be avoided due to interference of NSE from erythrocytes. The effect of sample type on results is not known. Biological variation is adequately studied in healthy individuals however not all studies give similar estimations. | [264,275,276] [277–283] |

| H-FABP (heart fatty acid binding protein) | | Function: involved in intracellular fatty acid transportation. Localization: Cytosolic protein | Early diagnosis of stroke. Potentially useful in stroke prognosis if used in combination with other markers. Several homemade and commercial assays (manual ELISAs and turbidimetric) have been used in studies but results may differ due to lack of standardization (lack of comparison studies). Knowledge on pre-analytical variables that may affect results is very limited. Age renal function body mass index and sex may affect levels. One small study has reported data on biological variation. | [284–288] [286,288–290] |

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<tr>
<td>Hemostatic biomarkers</td>
<td>vWF (von Willebrant factor) and its cleaving protease ADAMTS13</td>
<td>Function: Glycoprotein that stabilizes factor VIII, plays critical role in platelet adhesion and aggregation. Plasma vWF is mostly synthesized and secreted by endothelial cells under conditions of endothelial cell activation and damage.</td>
<td>High vWF (and low levels of ADAMTS13) have been associated with increased risk of stroke (not in all studies). Some studies indicate that levels of vWF may be a useful predictor of stroke subtype. Potential therapeutic target (using recombinant ADAMTS13). A variety of analytical methods to determine vWF activity or mass were used in different studies and the reporting vWF results in different units making the comparisons difficult. Commercial method is calibrated (using a WHO/NIBSC standard) and fine-tuned to detect vWF disease. The situation with ADAMTS 13 is similar. Both are characterized by high intraindividual biological variation that should be taken into account when interpreting serial results.</td>
<td>[96,113,114,293,294] [295–301]</td>
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<td>D-dimer</td>
<td>Localization: High molecular weight fibrinogen derivative, derived from the cleavage of cross-linked fibrin. It is generated through fibrinolysis during which fibrin polymers are cleaved by the enzyme plasmin. Function: Reflects both thrombin production and activation of fibrinolysis. It is a biomarker of thrombosis. In healthy individuals is not detected unless the coagulation system is activated.</td>
<td>It has been suggested as a prognostic marker of both IS and HS, risk of recurrent stroke and stroke progression. Plasma levels are associated with stroke subtypes and infarct volume. Low D-dimer levels are associated with early improvement. Standardization of assays that measure D-dimer remains an issue (lack of a calibrator that can be used to standardize the commercial assays currently available). There is also no consensus on how to report D-dimer results (at least 28 different combinations of measurement units are currently used to report D-dimer today), making comparisons among studies difficult, which underscores the need for global standardization of D-dimer result reporting. Influenced by age and concomitant medical conditions (heart failure, atrial fibrillation, CKD, and peripheral artery disease). Single biomarker measurements may be of limited utility due to large within-subject variation. D-dimer is stable for several months if stored properly.</td>
<td>[231,302–306] [307–314]</td>
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### Table 1. Continued

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<tr>
<td>Fibrinogen</td>
<td>Function: an acute phase protein involved in leukocyte–endothelial cell interactions, primary hemostasis, and platelet aggregation all of which play a central role in thrombus formation. Localization: Fibrinogen is synthesized (and secreted into the systemic circulation) primarily in the liver. Small amounts are also produced by endothelial cells. Elevated levels following acute IS associated with worse functional outcome and mortality. Risk biomarker for first and recurrent stroke. Known factors that affect fibrinogen levels are diet, use of several drugs, age, smoking, alcohol consumption, body mass, gender, physical exercise, race, genetic factors, and season. Fibrinogen is stable for several months if stored properly. There are several methods for measuring fibrinogen levels in plasma that may not give comparable results.</td>
<td>[315–319] [312,313,320–322]</td>
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<td>Fibronectin (cFn)</td>
<td>Function: Dimeric glycoprotein that serves cell to cell and cell–matrix interactions. Plays important role to clot formation by mediating the adhesion of platelets to fibrin. Localization: In blood circulate two forms: plasma fibronectin (p-Fn), which is primarily produced by hepatocytes, and cellular fibronectin (c-Fn), which is mainly synthesized by endothelial cells and circulates in small quantities. High plasma c-Fn levels are significantly associated with subsequent hemorrhagic transformation in stroke patients treated with tPA. High plasma levels are associated with malignant Middle Cerebral Artery infarction. Several research manual immunoassays available for its measurement but there are no data on their analytical validation. No data on pre-analytical requirements.</td>
<td>[323–325]</td>
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<td>CRP (C-reactive protein)</td>
<td>Function: Acute phase pentameric protein. CRP binds and aggravates a variety of soluble ligands. Activates the classical complement pathway. Localization: Found in blood, and produced in the liver in response to IL-6. Increased levels have been correlated with increased risk of stroke, recurrent stroke, infarct volume, stroke severity, and long-term outcome. Can serve as a prognostic factor for functional outcome in the early phase of stroke. Not all studies have found associations between increased CRP levels and stroke outcome or recurrent events. CRP is independently associated with the development of post-stroke infections. The optimal time-window for measurement is at 24–48 h. However, its additional predictive value is moderate over clinical information. Standardization is in progress however attention is needed when interpreting results from different assays. Age, sex, ethnic differences, and season of the year do not have an effect on CRP. However, exercise, smoking, obesity, alcohol, and medication may affect results.</td>
<td>[326–335] [336–339]</td>
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<tr>
<td>Adhesion molecules (VCAM, ICAM-1)</td>
<td>Function: Transmembrane immunoglobulin proteins involved in leukocyte-endothelial cell signal transduction. They mediate attachment and transendothelial migration of leukocytes an important event in stroke-induced inflammation. Their expression is Can be useful makers of stroke severity, recurrent stroke, and poor outcome. Not consistent results among studies. Single biomarker measurements may be of limited utility due to large within-subject variation. Commercial assays.</td>
<td>[311,340–343] [311]</td>
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<td><strong>Matrix Metalloproteinases</strong> (MMP-9, MMP-2) and their tissue inhibitors (TIMP-1, TIMP-2)</td>
<td>MMP-9, MMP-2</td>
<td>Induced under inflammatory conditions. Localization: soluble forms exist as a result of proteolytic cleavage from the cell surface and can be measured in biological fluids such as plasma, serum, or CSF. Function: It is a Zinc-binding proteolytic (matrix degrading) enzyme with critical role in CNS via BBB breakdown, demyelination, axonal injury and activation of inflammation.</td>
<td>Increased MMP-9 is related to BBB disruption. Predict hemorrhagic transformation (t-PA has been shown to directly activate MMP-9) and vasogenic edema formation. Plasma levels correlate to infarct volume, severity, and outcome. Increased after ICH. MMP-9 inhibition may be potential therapeutic target using TIMP-1 (its tissue inhibitor) as therapeutic strategy. Attention is needed in interpretation of results from different studies since sample type (serum or plasma) has significant influence on MMP-9 levels. Prolonged storage may compromise sample integrity (although a study found MMP-9 to be stable for at least 3 months if stored at &lt;4°C). Effect of ethnicity, age, gender, exercise, and diurnal variation not well studied. Significant intra-individual BV may limit its usefulness. Results from different kit manufacturers may differ.</td>
<td>[131,144,230,344–350] [311,351–356]</td>
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<td><strong>Lipoprotein-associated phospholipase A2</strong> (Lp-PLA2)</td>
<td>Lp-PLA2</td>
<td>Localization: produced by inflammatory cells (monocyte-derived macrophages, T-lymphocytes, and mast cells) and hydrolyzes oxidized phospholipids. Function: Lp-PLA2 is involved in the development of atherosclerosis depending on the type of lipoprotein particle with which the enzyme is associated.</td>
<td>May have a role in stroke prediction (primary and secondary) and outcome. Although the majority of studies show a positive association of Lp-PLA2 with both coronary and cerebrovascular disease, these associations have reduced strength after adjustment for well-accepted risk factors. Although the test that measures Lp-PLA2 has been approved by the FDA for the assessment of the risk of ischemic stroke and coronary disease, it is not widely used. There are two tests that measure Lp-PLA2, one measures the activity and the other the mass of the enzyme. Results from these two tests do not correlate well due to biochemical differences in the mass and activity tests.</td>
<td>[357–360] [361,362]</td>
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<td><strong>Cytokines</strong></td>
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<td>Localization: Soluble glycoproteins produced by brain cells in response to damaged tissue after ischemia. Microglia, astrocytes, neurons and endothelial cells are able to secrete pro-inflammatory and anti-inflammatory cytokines. An increase in production of pro-inflammatory and a decrease in production of anti-inflammatory cytokines is correlated with larger infarct size and worse outcome. TNF-α, and interleukins -1β, -6, and -10 are inflammatory.</td>
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<td>[363–371]</td>
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<td>Biomarker groups</td>
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<td>Function: regulate the innate</td>
<td>cytokines that have been found to be related to IS as prognostic biomarkers and as potential therapeutic targets. Variability of data on peripheral cytokines in human stroke is most likely due to the heterogeneity of stroke in</td>
<td>--------------------------------------------------------------------------------------------------------</td>
<td>[372–381]</td>
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<td>and adaptive immune response.</td>
<td>humans. Stroke severity, location, age of patients, comorbidities, and systemic inflammation prior to stroke may be key contributing factors to their post-stroke levels. The analysis of cytokines in blood samples may or may not reflect brain cytokine production. Pre-analytical and analytical variability may affect results. Direct measurement of free radicals is extremely difficult. Colorimetric and chromatographic research assays are available. No current consensus on which methods are the most useful, reliable, accurate or specific for different types of oxidative insults Despite successful application of these markers in some studies, analytical variability is a major concern for the evaluation of published studies. Little data is available on the pre-analytical and biological variability of OS markers in healthy individuals. Further in vivo studies assessing the variability and reliability of OS measures are needed. Copeptin is stable for days after blood collection (in EDTA plasma for up to 14 days at room temperature, while in citrate and heparin plasma copeptin is stable for 7 days) and can be quickly and easily measured with automated and manual immunoassays. So significant differences were observed in levels between CAPEPTIN levels.</td>
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Pre-analytical, analytical, and post-analytical concerns

Analytical validation of a laboratory assay usually refers to a number of characteristics that define the value of the assay. These can be related to the manufacturer of specific assay and to the analyte the assay tries to quantify.

The manufacturer analytical issues include accuracy, reproducibility, sensitivity, specificity, limit of detection, measurement range, recovery, high-dose-hook effect, interferences, etc. These characteristics will show how robust the assay is that we have used in our research.

The biomarker specific issues are mostly pre-analytical. Most studies evaluating new biomarkers use patient samples that are stored for long periods and collections are made more than once in each patient. For the majority of biomarkers that have been tested in clinical studies, the requirements for long term are not known since there is a lack of studies that examine their stability under long-term storage conditions. Our knowledge is also limited on which type of biological sample is most suited for analysis (blood, urine, CSF etc.), and when we collect blood, if it is better to use serum or plasma. In order to make better use of serial collections and interpret changes in the concentration of a specific biomarker, we need to know its biological variation (ideally in both health and in disease). In the literature, few studies deal with this issue (Table 1).

Standardization of biomarker assays

Unfortunately, for the majority of biomarkers tested so far in most research studies the laboratory assays that have been used are in their very early stages of their development. These are either in-laboratory developed assays or in the best case commercially available but research-use-only assays (RUO). That means the results that the various clinical studies have produced cannot be compared.

To achieve comparable results, all measurement procedures must measure the same quantity. In addition, the calibration of all measurement procedures should be traceable to a common reference system consisting of reference methods and materials. The process for achieving these requirements is commonly referred to as “establishing metrological traceability”. The terms “harmonization” and “standardization” are used to describe the two principal approaches for establishing metrological traceability. The term “standardization” is used when all of the following conditions are met:

- the clear definition of the analyte that we are going to measure,
- there is a higher order reference measurement procedure (RMP) and primary reference materials (PRM) which is actually a calibrator that will contain the analyte in its pure form,
- the agreement of test results is achieved by establishing traceability to these by using the International System of Units (SI),
- and finally, the existence of an external quality assessment scheme that laboratories can participate.

Unfortunately, none of the above conditions can be met for the majority of biomarkers under investigation. Higher-order RMPs are well-characterized analytical methods that are intended for assigning target values to PRM and have a higher level of accuracy, precision, and specificity than the commercial measurement procedures. This is the preferred way to produce harmonized results through a standardization process.

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<td></td>
<td>Plasma</td>
<td>plasma and serum. Diurnal variation in healthy controls is limited with slightly higher values in the morning. Copeptin levels were found to be higher in the male volunteers compared with female. Especially in men, there is a strong relationship between copeptin and decreased glomerular filtration rate, probably due to decreased renal copeptin clearance. Furthermore, corticosteroids appear to inhibit copeptin release. Physical activity moderately affects levels. The assay needs standardization.</td>
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<td>Table 1. Continued</td>
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Primary reference material, reference methods, and secondary reference material are now available for many of the most important analytes determined by conventional chemical methods, but immunoassay standardization is less well developed [202–206].

However, the standardization of measurements of plasma proteins has been proved a difficult task. They are potentially heterogeneous molecules, in the systemic circulation we can find several fragments of various molecular weight, they are usually subject to post-translational modifications, such as glycosylation and substitution, and finally, they exhibit differences in primary, secondary, tertiary, and quaternary structure. However, they present as a target for standardization due to their importance in laboratory diagnostics and health care and because of the observed significant discrepancies in measurement results obtained with non-standardized assays [207].

What happens when there is no reference procedure and no certified primary reference material? If standardization, as described above, cannot be achieved, agreement among measurement procedures can still be obtained through the process called “harmonization”, in which there is a reference system consisting of methods and materials that are not traceable to the SI but “act as references”. One or more methods can be selected as “designated comparison method” and can be used to assign values to one or more reference materials. The terms “all-method trimmed mean” and “all-procedure trimmed mean” are also used to describe this summary statistic by which the assignment of value is done. Reference materials can be prepared from purified biomarkers, a set of single-donor blood materials, or pooled patient samples. In special cases, a manufacturer’s calibrator can be designated as a reference material. Both standardization and harmonization activities aim to achieve comparable and reliable measurement results. Whereas standardization ensures traceability to the SI, harmonization ensures traceability to a reference system agreed upon by convention [202,206,208,209].

Can old measurements of the biomarker from clinical and epidemiological studies be calibrated (standardized) to the gold standard reference measurement procedures? Yes – if properly stored serum samples are available and a statistical procedure is defined. Such calibration studies are usually used to convert values from old or discontinued laboratory methods to the values obtained from new or currently used methods. An extension of this concept is to calibrate values from old or discontinued laboratory methods to the “true” values that are obtained using a “gold standard” laboratory method commonly referred to as reference measurement procedure [202]. A paradigm comes from the vitamin D standardization program [210–212].

**What is produced locally is also reflected systemically? – influence of BBB**

Many studies have shown that in pathological conditions such as acute stroke, BBB integrity is compromised and followed by the extravasation of blood components into the brain disturbing the neuronal function. On the other hand, brain-derived proteins enter the systemic circulation. However, the physiological activity that allows neuronal and glial factors to reach the peripheral circulation remains largely undetermined. There are two general hypotheses trying to explain how brain-derived proteins enter the bloodstream. First, brain injury causes damage to the neurovascular unit (NVU), particularly at the capillary level, and this may allow CNS proteins to cross the BBB. Alternatively, brain-derived proteins may be cleared from the CNS after injury, by the lymphatic system. The glymphatic system is a recently discovered waste clearance system that utilizes a unique system of perivascular channels, formed by astroglial cells, to promote efficient elimination of soluble proteins and metabolites from the central nervous system [213,214]. Moreover, several other patient characteristics such as age and sex comorbidities may influence the BBB permeability [213,215,216]. The question is if the levels of the brain-derived proteins that we measure in the systemic circulation reflect their production in the brain. The BBB delays the passage of the various substances released from glial cells and neurons to the systemic circulation. Even when the BBB is disrupted by stroke, hypoperfusion of the infarcted tissue may obstruct their release into blood. Another problem is how specific are these proteins for cerebral ischemia, since several other etiologies of brain dysfunction may produce similar BBB dysfunction and release of similar molecules. Finally, the brain is comprised of heterogeneous cell populations that differ in composition depending on the region. Functional deficits may be more a function of location than of size of tissue injury. Therefore, a small area of ischemia in a specific region of the brain may cause disproportionally higher symptoms than a bigger ischemic area in a different location [178,217,218]. Alterations in the integrity of the BBB can alter the ability of CNS molecules to enter the systemic circulation. A recent study that examined BBB function after stroke concluded that lacunar stroke patients had widespread BBB disruption and indicated that the relationship between BBB damage and infarct size is not proportional [219]. The extent of BBB integrity and the size of brain tissue damage are not strictly correlated. This implies that serum concentrations of CNS biomarkers may not correlate with the absolute values of the same biomarkers in the CSF [220]. Marchi et al. have
shown the influence of BBB in the serum levels of brain-derived biomarkers [126,221]. Biomarkers can also pass into the systemic circulation even when the BBB is intact and most of the neuronal biomarkers have been detected in the serum of healthy individuals. The ability of some of these biomarkers to cross the BBB and enter circulation seems unrelated to its function and integrity and must be mediated by the solubility of the molecule and its molecular weight. The interpretation of biomarker levels is challenging especially when we want them to be able to distinguish between BBB dysfunction alone, co-current BBB dysfunction and tissue damage or substantial tissue damage with BBB in good condition [199].

**Patient and disease heterogeneity**

In complex conditions, such as stroke, there is a large degree of heterogeneity between patients. For example, biochemical profiles are very likely to be altered by the site, intensity, and duration of ischemia in acute ischemic stroke. The additional effects of patient sex, age, ethnicity, and possible comorbidities, limit the comparability between studies. Therefore, it seems logical the same biomarker to perform differently in different patient cohorts [222].

Another major problem in biomarker discovery and validation is disease heterogeneity. Advances in molecular analysis methods have revealed that even diseases that were considered monotypic in the past incorporate multiple molecular forms that share a common clinical presentation. Stroke is the perfect example of a heterogeneous disease. It is therefore logical to hypothesize that disease heterogeneity may be responsible for the considerable variability in sensitivity and specificity that was observed in various studies when the same biomarker was under investigation. Heterogeneous diseases may not have a single biomarker that is predictive for all patients. Each subtype may have its own biomarker or set of unique biomarkers. That complicates furthermore biomarker interpretation. Co-morbid processes can alter certain biomarker levels. Correlations between biomarker levels and disease severity or outcome are generated by demonstrating a difference in the mean levels between different groups with different clinical status. However, a concomitant infection in a certain patient may increase cytokine and other inflammatory markers levels despite a small infarct, which will confound their correlation with infarct size. Many inflammatory biomarkers, especially cytokines cannot be interpreted without taking into account the complex role they play in their highly regulated systems.

**Effect of sample size in research studies**

In many studies, the sample size tested is small, making it difficult to establish the true relationship between a marker and patient diagnosis or prognosis. The analysis of samples from large patient cohorts, stratified by known risk factors, should minimize the influence of clinical confounding variables. A recent article has commented that small sample studies present the risk to inflate biomarker associations their result must be interpreted with caution [223].

The heterogeneity of stroke also introduces challenges for research studies that focus on the discovery and validation of new biomarkers because of the need of adequate sample size in order to ensure that all relevant subtypes are adequately represented in the study. But what is the right sample size? How is it calculated? Do we need a different statistical approach and if so which one? Recently, Wallstrom et al. tried a theoretical approach to answer these key questions, however, these issues have not been examined in detail and most statistical analyzes that will determine the sample size requirements do not take into account the disease heterogeneity [224].

**Single biomarker versus biomarker panels**

Given the heterogeneity of ischemic stroke, a single biomarker may not be sufficient to reflect the underlying complexity. Several potential clinical applications have been considered for biomarkers (risk for the development of the disease, diagnosis, characterizing clinical severity, identifying ischemic penumbra, estimating risk of progression or worsening, and outcome). Many biomarkers that have been examined are associated with brain ischemia, but they offer little additional information for the individual patient beyond that obtained from clinical examination and neuroimaging.

In an attempt to address these limitations, more complex models were tried involving the simultaneous measurements of several biomarkers. In some studies, even 50 different biomarkers were measured at the same time. These panels were created in order to incorporate biomarkers that reflect pathophysiological processes of the disease like atheromatosis, thrombus formation, inflammation, oxidative stress, endothelial injury, BBB disruption, and cerebral ischemia. The strategy in these studies was to combine sensitive but not tissue-specific biomarkers with at least one tissue-specific marker. Very few biomarkers reached sensitivity and specificity over 90%. Even the addition of a few basic demographic data (like age sex or the presence of atrial fibrillation) did not improve performance significantly [225–231].
What is the best way to select biomarkers to include into a panel? This is an active field of investigation in the field of bioinformatics [232,233]. One approach is to include all those biomarkers that are significantly different in patients compared to controls and try to combine them in order to build a predictive model. However, very often we can find two biomarkers that provide similar clinical information and are significantly different between the compared clinical groups. Therefore, it is imperative to include markers that combine well together as predictors. For this, a number of techniques have been developed. Once the biomarkers are selected there are several ways and methods to assemble them into a prediction model. Once the prediction model is generated, it must be evaluated in a different cohort in order to avoid bias. Recommendations for the development and evaluation of biomarker panels have been published [234].

**Single biomarker versus multiplexed assays**

In most of the studies, investigators have used Enzyme-Linked Immuno-Sorbent Assays (ELISA) for the measurement of several biomarkers (especially cytokines), as a quantification tool. Recently, more rapid and automated assays based on the same principle have been developed. These so-called multiplexed immunoassays have the ability to quantify multiple cytokines simultaneously, with the advantage of using small amounts of sample and faster turnaround times [235–240].

ELISA has its roots back in the late 1960s [241–245]. This method enables accurate and reproducible quantification of the antigens of interest. ELISA has become the standard tool in most research studies involving the measurement of protein biomarkers. Immunoassays make use of the specific, high-affinity interaction between an antigen and its antibody. Two major types of assay formats have been used for immunoassays. The so-called competitive and non-competitive or sandwich formats. The sensitivity of the assay may be enhanced by the use of signal amplification steps that may involve either enzymes or the use of avidin–biotin complexes. Although ELISA is a very valuable research tool, there are several weaknesses associated with this technique [246].

Variations between different kits exist and are mainly due to recognition profiles of the antibody pairs manufacturers use in their immunoassays. This can be addressed by the better characterization of the antibodies [247]. The calibrators used in the different experimental immunoassays are almost always recombinant proteins that were produced using various “systems” including *Escherichia coli*, yeast, and mammalian cells. These proteins do not entirely represent the complex and heterogeneous mixture of natural proteins found in the systemic circulation and in other biological fluids which may contain precursor molecules, fragments, different isoforms etc. The antibodies that are used in these assays are also made against these recombinant proteins. The performance is largely dependent on antibody quality, i.e. affinity, specificity, and avidity, the selection of appropriate blocking and dilution buffers, and the incubation periods [246].

Other significant problems include interferences from autoantibodies and other binding proteins that may be present in the biological sample. As with all immunoassays, ELISAs are also prone to cross-reactivity and matrix-effect. Cross-reactivity is the possibility of the antibodies binding to more than one antigen, thereby causing an erroneous result and often a false-positive effect. Another problem is interferences that result from the matrix effects of the samples, which is due to unknown and unspecified factors present in the sample that interfere with the immunoassay. Matrix effect is usually manifested by low recovery of a specific amount of cytokine spiked into a sample or a non-linear dilution of the sample. This can be resolved by preparing the calibrators in a solution that closely resembles the sample, however, the selection of such diluent is not a simple task. In any case, commercial assays should only be used with samples types that these kits are validated for in order to minimize matrix effect. However, this cannot be said with certainty because investigators use homemade assays in their studies. These studies should be used with caution in reviews and meta-analyses.

Other weaknesses of traditional ELISA include that it lacks sensitivity, it is relatively time consuming, it requires relatively large quantities of sample, and it can only detect one molecule in a single experiment. Another limiting factor of ELISA is that the colorimetric reagents are used for the final reading, which may reduce the dynamic range for these assays.

Finally, we must not forget that all ELISA is a manual method and its performance is highly dependent on operators’ skills and experience. User errors and reagent stability are the two major factors affecting the reproducibility of an assay [248–250].

The technological developments in the last decades have resulted in the discovery of multiplex platform assays which allow for the simultaneous quantification of multiple cytokines in a single aliquot of specimen [251]. Several companies have produced multiplex platforms based on flow cytometry chemiluminescence and electrochemiluminescence technologies. The popularity of these multiplex platforms has increased in the last decade especially for the measurement of cytokines, since they permit the simultaneous measurement of up
to 25 cytokines in the same sample. In several situations, this number can be exceeded. The advantages over classic ELISA include high-throughput multiplex analysis, less sample volume, efficiency in terms of time and cost, ability to perform repeated measurement to the same patient of the same panel of cytokines under the same conditions [235,251,252].

Contemporary multiplex immunoassay systems may be divided into two categories: planar assays and microbead-based suspension assays. The first includes platforms such as the Mesoscale discovery Technology Platform (MSD), the Q-plex array (Quansys Biosciences, Logan, UT), and the Biochip Array Technology (Randox Laboratories). With this technology, high-affinity capture ligands are immobilized discretely on a solid phase. The immobilized ligands are subsequently exposed to treatment with the sample under investigation and probing with detection antibodies labeled with a reporter system. The suspension format includes platforms like Luminex, Cytometric Bead Arrays, and Bioplex Pro (Bio-rad Laboratories). Here high-affinity ligands are immobilized discretely on fluorescently activated plastic microbeads and mixed with the sample in the liquid phase. Subsequently, the addition of detection antibodies labeled with a reporter dye enables high-resolution analysis of specific fluorescent signal via flow cytometric methods.

Pitfalls still exist with these multiplexed immunoassays, with lack of standardization, interferences, specificity of capture antibodies, and comparability between different platforms to be on top [235,252–258].

While it is evident that multiplexed immunoassays hold great promise for cytokine profiling, there are still important issues requiring further studies. The heterogeneity of the immobilized sample necessitates the use of highly specific antibodies to eliminate false positive responses, universally optimized sample diluents, uniformly calibrated standards with mass values and internal assay controls. This should greatly facilitate intra-laboratory accuracy and precision and external quality assessment schemes that improve the inter-laboratory comparisons of cytokine measurements.

Conclusions: let us put the horse in front of the cart

Conclusions arising from numerous research articles, meta-analyses, and reviews (systematic or narrative) published over the last 10 years in this field vary greatly leading to a considerable difference of opinion on the clinical applications of stroke biomarkers. The key questions if biomarkers can be used in stroke diagnosis, differentiation, progression, prediction of outcome, monitoring of therapy etc., or what is the added value of the biomarkers to current standard clinical practice are always answered with a not so enthusiastic “maybe but not yet”. It is true that stroke is an extremely heterogeneous disease that affects patients that are also heterogeneous themselves with various comorbidities. However, researchers designed clinical studies that required the measurement of biomarkers with non-standardized assays and their results cannot be compared. Therefore, meta-analyses and reviews are of very limited value if at all.

The use of unstandardized assays in clinical research can offer only confusion and incorrect conclusions to researchers. It is worth to point out again the example of how vitamin D assay standardization has changed the results of the NHANES study. This is a very good example of how the use of unstandardized assays in clinical studies can lead clinical scientists to wrong results.

So let’s put the horse in front of the cart in this case. To leading clinical and laboratory organizations dealing with stroke, we propose the following:

- A team of scientists to select a number of biomarkers that will show promise to standardize (or at least to harmonize) using an agreed and standardized method;
- These biomarkers must fulfill certain criteria (i.e. reflect not only pathophysiological processes but also show promise for laboratory automation);
- A proposal to IFCC to start a standardization (or harmonization process) with involvement of IVD industry;
- We must find prospective biomarker studies that have stored samples “recalibrate their assays” to the new assay by a standardized protocol, and re-examine the results; and
- Proceed to new prospective studies.

It is our belief that if we do not proceed this way we are not going to eliminate the biggest confounder from our clinical studies: the laboratory assays.

Disclosure statement

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