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Blood biomarkers to guide stroke thrombolysis

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1. ABSTRACT

The use of specific brain biomarkers might aid stroke diagnosis and that approach might permit rapid referral of stroke patients to hospitals with acute treatments, such as t-PA being available. Although thrombolytic therapy in acute stroke is effective since it accelerates clot lysis and earlier restoration of blood flow, many treated patients do not recanalize or do it too late and some suffer hemorrhagic transformations (HT) with high death rates. It has been recently described that biomarkers such as MMP-9 or fibronectin, might be used to select patients at higher risk of HT, and high PAI-1 that interferes with tPA-induced recanalization, might predict clot-lysis resistance and poor outcome. Moreover, high levels of MMP-9 and MMP-13 are involved in infarct growth in spite of thrombolytic therapy suggesting its ultra-early role in brain injury. Other biomarkers such as CRP may predict stroke mortality following reperfusion therapies. We will also show that the genetic background of stroke patients might condition plasma levels of some of those biomarkers and influence the therapeutic response in t-PA treated patients.

2. PLASMA BIOMARKERS TO IMPROVE STROKE DIAGNOSIS

Decision making processes using biomarkers are widely accepted in several medical situations. In recent years, new approaches have been identified for plasma biomarkers in the acute stroke field such as the idea of producing a biochemical stroke diagnosis or using them as prognostic point-of-care tools. The case of stroke thrombolysis in which very quick decisions have to be taken by clinicians seems an excellent scenario to test the utility of emerging brain biomarkers.

At present, the absence of a widely available diagnostic test for acute cerebral ischemia remains a limitation in the diagnosis and management of stroke (mostly made on clinical grounds and Computed Tomography (CT)). Therefore, several authors are trying to evaluate the prognostic value of diagnostic panels of blood-borne biochemical markers of cerebral ischemia. Although the ideal scenario for

specific brain biomarkers is to differentiate "real strokes" from "other causes mimicking stroke (mimics)", until now research work has mainly focused on biomarkers that distinguish stroke patients from controls. Biomarkers tested in that kind of studies have included: S-100B (a marker of astrocytic activation), B-type neurotrophic growth factor, von Willebrand factor, matrix metalloproteinase-9, and monocyte chemotactic protein-1. In a panel algorithm in which three or more marker values above selected cut-offs were scored as positive, these five markers provided a sensitivity of 92% at 93% specificity for ischemic stroke samples taken within 6 h from symptom onset (1). Another study from the same group, analyzed 26 blood-borne markers, and revealed that 4 were highly correlated with stroke ($P<0.001$): a marker of glial activation (S100B), 2 markers of inflammation (matrix metalloproteinase-9 and vascular cell adhesion molecule), and 1 marker of thrombosis (von Willebrand factor). The logistic model provided a sensitivity and specificity of 90% for predicting stroke (2).

These data suggest that the use of a combination of biomarkers seems useful in making an urgent biochemical diagnosis of stroke. If true, this approach would permit rapid referral of stroke patients to hospitals with acute treatments available.

Proteomics is also a promising tool for large-scale biomarker identification in the stroke field, mainly directed to diagnosis and treatment response. In fact, several biomarkers have been identified from 2-dimensional electrophoresis gels of post-mortem CSF, which were compared with gels from ante-mortem CSF in a different set of patients, used as model of massive brain damage (3). In that study, the identification of PARK7 and NDKA was based on their increased concentrations in post-mortem CSF, presumably as a result of global brain ischemia and necrosis after death.

Moreover, biomarkers might be of additional use in the differentiation of stroke subtypes. Although brain CT is performed as part of the initial evaluation of a patient with suspected stroke in most hospitals, because of its high sensitivity to differentiate ischemic from hemorrhagic stroke, the availability of a rapid biochemical diagnostic test would add clear advantages in the management of stroke patients. Some authors have already described biomarkers such as glial fibrillary acidic protein (GFAP) (4), which was substantially raised in patients with ICH, making these kinds of tests very attractive for pre-hospital assessment.

Also low soluble form of the receptor for advanced glycation end-products (RAGE) and high S100B was identified among ICH as compared to ischemic stroke by our group (5) and low ApoC-I and ApoC-III was found by others (6). In that study, ApoC-I and ApoC-III levels provided very good predictions of ischemic versus hemorrhagic stroke (94% sensitivity and 73% specificity for ApoC-I and 94% sensitivity and 87% specificity for ApoC-III). All these markers need confirmation and more precise studies in the future.

3. BIOMARKERS AND SYMPTOMATIC HEMORRHAGES APPEARANCE FOLLOWING T-PA

Intravenous recombinant tissue plasminogen activator (t-PA) administered within 3 hours from symptom onset has been proven to be an effective therapy for acute ischemic stroke (7). However, its use in clinical practice is not generalized worldwide (8). The most feared complication of thrombolytic therapy is symptomatic intracranial hemorrhage (SICH), and it is probably the main reason that, at present, only a small fraction of potentially eligible stroke patients are treated with t-PA.

Although t-PA is associated with fewer hazards and more benefit, the optimum criteria to identify the patients most likely to benefit, the latest time window, and the potential risk of increasing age, stroke severity, dose, and route of administration are not clear. Whether biomarkers may aid in these grey areas is outside the scope of this review.

Previous studies pointed out clinical (stroke severity, old age, high blood pressure) radiological (early CT changes, T2* microbleeds), and sonographic (proximal middle cerebral artery occlusion), predictors of hemorrhagic transformation (9-13). A particular interest has emerged as to the use of analytical or biochemical markers as pre-treatment predictors of SICH. Recent reviews have covered some parts of this issue in experimental cerebral ischemia (14), therefore we will try to summarize the actual knowledge on molecular mechanisms and surrogate biomarkers that might be useful in the diagnosis and treatment of t-PA induced brain bleedings following human stroke.

Hemorrhagic transformation in ischemic stroke is preceded by damage within the neurovascular unit, which comprises the cerebral endothelial cells, astrocytes and neurons, in a milieu of extracellular matrix (ECM) (15, 16).

Locally-formed oxygen free radicals and activated plasmin lead to the secretion of proteinases in the ECM, which degrade the components of the basal lamina of the blood brain barrier (BBB), causing vasogenic edema and extravasation of blood into the brain parenchyma (17, 18). Destruction of microvascular integrity is a result of the degradation of the basal lamina and extracellular matrix (19). Matrix metalloproteinases (MMPs) are thought to be key molecules in this process and recent data demonstrates that exogenous t-PA may exacerbate

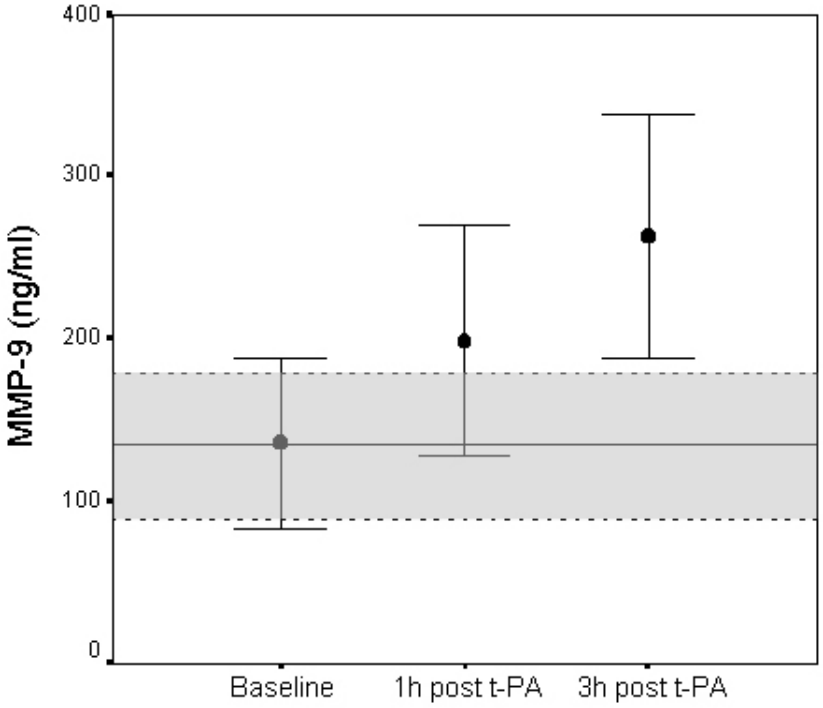

Figure 1. Increase of MMP-9 induced by thrombolytic treatment. Error bars represent plasma MMP-9 level among patients who received t-PA and the grey area indicates reference interval for controls (stroke patients who did not receive t-PA). t-PA induced MMP-9 peaked one to three hours after treatment onset. Modified with permission from reference 29.

BBB breakdown by contributing to MMP activation, which mediates t-PA's extra-vascular adverse effects.

MMPs, the largest class of human proteases, have been implicated in both animal and human studies of t-PA-related hemorrhagic transformation. MMP-2 and MMP-9 (gelatinases) specifically attack type IV collagen, laminin, and fibronectin, the major components of the basal lamina around cerebral blood vessels (20).

Lo and colleagues found that knock-out mice deficient in MMP-9 expression were protected against BBB injury and had better post-stroke motor recovery than controls (21). Moreover, Rosenberg and others found that injection of MMP-9 increased infarct size, while broad-spectrum MMP-9 inhibition reduced both the volume of infarct and the rate of hemorrhagic conversion (22, 23). A more recent study used a rat and mouse model of focal cerebral ischemia to investigate the relationship between t-PA and MMP-9 *in vivo*. Administration of exogenous t-PA doubled the "normal" MMP-9 response after ischemia in rats, t-PA gene knockout significantly decreased ischemic MMP-9 levels compared with WT mice, and exogenous t-PA reinstated the MMP-9 response back up to WT levels. Together, these pharmacological and genetic data show that t-PA can amplify MMP-9 in stroke *in vivo* (24).

Regarding humans, plasma levels of total MMP-9 corroborate the *in vivo* animal studies. MMP-9 levels are elevated in patients with acute ischemic stroke (25), and the degree of elevation predicts cerebral ischemic hemorrhagic transformation (26, 27). More importantly, we have also shown that pre-t-PA treatment MMP-9 levels may predict thrombolysis-related injuries (28). A higher baseline MMP-9 level (measured at patients arrival at the emergency department) was observed in patients with SICH (289.0 ng/ml) compared with patients with non-symptomatic hemorrhages or without hemorrhagic transformations (119.5 ng/ml, p=0.020). Baseline MMP-9 levels Odds Ratio (OR) 9.62 (1.31 to 70.26; p=0.025) remained as the main independent predictor of the appearance of a parenchymal hemorrhage following t-PA treatment. Moreover, in stroke patients treated with thrombolytics a peak in plasma MMP-9 levels was observed after 1 to 3 hours following t-PA infusion (29) as shown in Figure 1. This peak was prominent among patients who later on displayed a hemorrhagic transformation.

Altogether, these data suggest that MMPs may be a promising marker of stroke outcome, since some of those (MMP-9 and MMP-13) are very well correlated with infarct expansion evaluated by Diffusion-Weighted Image (DWI) MRI and have the

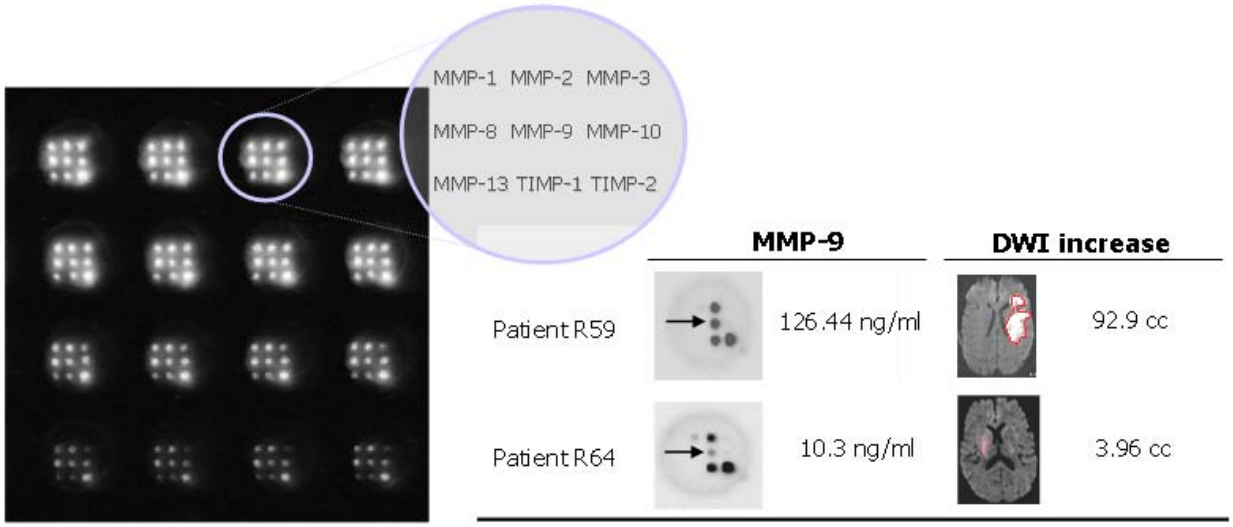

Figure 2. Metalloproteinases and brain infarct extension relationship. A protein array for matrix metalloproteinases allows multiplexing nine different biomarkers in the same plasma sample. This example shows high MMP-9 in a patient that developed a larger infarction. Modified permission from reference 30.

potential to guide triage for thrombolysis therapy (30) . Multiplexing techniques such as those shown in Figure 2 will allow measuring many of those biomarkers at the same sample.

Fibronectin is another interesting biomarker with potential diagnostic benefit. Fibronectins are adhesive dimeric glycoproteins that promote cell–cell and cell–matrix interactions (31). Plasma fibronectin (p-Fn) is primarily produced by hepatocytes, but plasma also contains small quantities of cellular fibronectin (c-Fn), which is mainly synthesized by endothelial cells (32). Because c-Fn is largely confined to the vascular endothelium, high plasma levels of this molecule might be indicative of endothelial damage. A recent study demonstrates that plasma c-Fn levels are significantly higher in patients in whom HT develops after t-PA administration and suggests that c-Fn leve ≥3.6 µg/ml can predict the development of HI-2 and PH after t-PA administration with a sensitivity and negative predictive value of 100% (33). Recently a multi-centric study confirmed the value of MMP-9 and fibronectin predicting HT after stroke thrombolysis (34).

Another very large multi-centric study identified elevated S100B serum levels measured before thrombolytic therapy as an independent risk factor for HT in patients with acute stroke. Unfortunately, the diagnostic accuracy of S100B was too low for it to function in that context as a reliable biomarker in clinical practice. (35)

After an acute ischemic event, the release of endogenous inhibitors of fibrinolysis into the blood flow (36, 37) might lead to wide inter-individual differences in the global fibrinolytic capacity, which are able to block or enhance t-PA effects playing a key role in development of SICH. To test that hypothesis we determined the impact of pretreatment levels of several fibrinolysis inhibitors (plasminogen activator inhibitor (PAI)-1, lipoprotein a (Lp (a)), thrombin-activated fibrinolysis inhibitor (TAFI), and homocysteine on the development of SICH (38).

When laboratory data were analyzed, patients who experienced SICH showed lower baseline PAI-1 (21.7±3.5 ng/ml versus 31.8±12.1 ng/ml; P<0.01) and higher TAFI (216.7±78.4% versus 162.1±54.2%; P=0.03). The combination of admission PAI-1 <21.4 ng/ml and TAFI >180% had a sensibility of 75% and a specificity of 97.6% (P<0.01) predicting SICH, with a positive predictive value of 75% and negative predictive value of 97.6%.

A recent report could not extend these results to other haemostatic parameters such as fibrinogen, prothrombin fragments 1+2, Factor XIII, Factor VII, alpha2 anti-plasmin and functional TAFI (39).

Low platelet counts and anti-platelet therapy (9) have been associated with SICH after tPA treatment, suggesting that the coagulation/fibrinolytic balance is implicated in the bleeding process. Moreover, fibrin (ogen) degradation products could also predict hemorrhagic transformation after thrombolytic therapy (40). Very recently the plasma protein profiles of acute stroke patients who developed ICH after t-PA versus those who did not was examined (41). The authors characterized plasma protein profiles by Surface Enhanced Laser Desorption/Ionization (SELDI) analyses identifying

the intensities of 11 protein peaks in the low molecular weight spectra (fewer than 20,000 Daltons) that were significantly different (8 increased and 3 decreased) in the plasma from ICH versus non-bleed patients at 24 hours. Additionally, at baseline, one peak (4412.979 m/z) was already significantly increased in ICH versus non-bleed patient plasmas. Some of these proteins included apolipoprotein C1 and serum amyloid A, and others are being identified. These results demonstrated that several plasma biomarkers may potentially identify patients at risk to develop ICH after t-PA administration for acute ischemic stroke prior to treatment although results are still far from clinical practice.

Since the response to t-PA may be strikingly different among similar patients, ranging from a total reopening of the occluded artery with complete neurological recovery to the appearance of a huge hemorrhage into the infarct territory, we hypothesized that a genetically predetermined risk for clinical response after thrombolysis might also exist in some stroke patients.

GENO-t-PA is a study conducted by several groups to test genetic markers of safety and efficacy among stroke patients receiving t-PA. In that study we have identified a polymorphism in FXIII gene related with unacceptable rates of brain bleedings (42). As a result of that link the V34L polymorphism was associated with mortality: 20.0% of L34 carriers but 9.1% of patients with V/V genotype died after thrombolytic therapy (P=0.034; OR=2.50).

4. LACK OF RECANALIZATION, REPERFUSION INJURY AND VESSEL REOCCLUSION

The fibrinolytic balance is also relevant for brain vessel recanalization. In fact, Ribo *et al*, showed that endogenous inhibitors of fibrinolysis may be involved in t-PA resistance, decreasing stroke thrombolysis benefits (43). In that study, the impact of pre-treatment levels of PAI-1, lipoprotein (a), TAFI and homocysteine on arterial recanalization was evaluated. After a regression model was applied the only independent predictor of thrombolysis resistance was baseline PAI-1>34 ng/ml.

In a similar study, patients who recanalized had lower concentrations of alpha2-antiplasmin (87.5% vs 96.5%, p =0.023) and functional TAFI (91.7% vs 104.4%, p = 0.039) (44). However, the multi-variant logistic regression analysis showed that the level of alpha2-antiplasmin was the only predictive variable of recanalization (OR 0.95, 95% CI 0.91, 0.99, p = 0.038).

Following a successful recanalization, reperfusion injury and reocclusion are other possible vascular complications among t-PA treated patients. However, almost no data is available about both phenomena and no biomarker has been tested in order to predict its appearance. Since preliminary data indicate that as many as 12% of t-PA treated patients might suffer reocclusions of t-PA reopened vessels (45), it will be very interesting to explore whether biomarkers that are prone to modifications following vessel recanalization or thrombolysis failure might be good candidates to predict reocclusion and thereafter to initiate more aggressive therapies in that subset of patients.

For example, a single S100B value <0.4 µg/l obtained 48-96 hours after stroke onset indicates successful clot lysis <6 hours in MCA/M1 occlusion with a high degree of accuracy (46). Thus, those authors suggested that determination of a single S100B value may serve as a surrogate marker of early and sufficient MCA/M1 recanalisation in large scale thrombolytic studies. Also, MMP-9 plasma levels were proposed by others as an index of thrombolysis failure since significantly higher levels of these biomarkers were found in patients whose arteries were not recanalized (47).

5. CLINICAL WORSENING AND MORTALITY

Selected biomarkers have been shown to be useful to predict neurological worsening (IL-6) following ischemic stroke (48). Many of those biomarkers are linked to neuroinflammatory cascades (49) and are potential candidates which could be tested in t-PA treated patients.

A recent study of our group demonstrated that admission (<3h) high sensitivity C-reactive protein (CRP) predicts mortality after t-PA treatment for stroke patients, adding prognostic information to classical risk factors (50). This is of clinical importance because CRP is a powerful marker that might be used as a point-of-care tool for the risk stratification of stroke patients' candidates to receive t-PA.

Together with high CRP, advanced age is another independent predictor of mortality following t-PA. The risk of death following t-PA reached 54% in patients older than 72 years who were in the upper CRP quartile. Therefore both factors (age and CRP) might interact negatively. Although some studies has demonstrated that t-PA is a safe and effective treatment among old stroke patients (51), the European Agency for the Evaluation of Medicinal Products has only approved the treatment for those under 80. Therefore, age is still a concern that might be overcome adding biomarkers, such as CRP, to the decision of giving t-PA to old patients.

Malignant MCA infarction has a very bad prognosis with huge mortality rates. Föerch and colleagues identified 12-hour S100B value >0.35 microg/L as a predictor of malignant infarction with 0.75 sensitivity and 0.80 specificity (52). Since the serum marker S100B can predict a malignant course of infarction in proximal MCA occlusion, authors propose identification and monitoring of patients at particularly high risk for herniation using biomarkers.

Similarly, Serena *et al*, conducted a study to determine whether molecular markers of endothelial damage may help to predict secondary brain oedema and, to identify patients who could benefit from aggressive therapies such as decompressive hemicraniectomy or hypothermia (53). They measured plasma concentrations of glutamate, glycine, gamma-aminobutyric acid, interleukin-6 (IL-6), IL-10, tumor necrosis factor-alpha, MMP-9 and cellular-fibronectin (c-Fn) at admission, showing that c-Fn >16.6 microg/ml had the highest sensitivity (90%), specificity (100%), and negative and positive predictive values (89% and 100%, respectively) for the prediction of malignant MCA infarction.

To summarize this data, we may conclude that in the near future, biomarkers discovery might identify candidate proteins to make a diagnosis of ischemic stroke and to predict complications following t-PA treatment in order to increase thrombolysis safety and efficacy. Therefore, post-stroke biomarker profile might help to determine the best reperfusion strategy for every individual patient. For that reason, researchers and companies need to make an effort in developing point of care devices that allow translation of the exposed findings to daily practice.

Moreover, when new useful biomarkers are identified, their pathways and mechanisms in the ischemic cascade should be dissected since these findings might guide us to new therapeutic interventions and drug targets.

6. ACKNOWLEDGEMENT

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