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Neutrophils in tPA-induced hemorrhagic transformations: main culprit, accomplice or innocent bystander?

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ABSTRACT

The risk of intracerebral hemorrhage still greatly limits the use of tPA in stroke patients. Research is ongoing in order to identify the pathophysiological mechanisms at play, detect predictive biomarkers and discover new pharmacological targets to develop preventive or curative treatments. Going through experimental and clinical studies, this review focuses on the role of neutrophils as key predictive biomarkers for thrombolysis-induced hemorrhages and as pharmacological targets to limit their occurrence. To date, there are no established pharmacological modulators of neutrophils for ischemic stroke and its hemorrhagic complications. Several strategies are under evaluation, including lipid-lowering drugs, free radical scavengers, or minocycline, as well as non-pharmacological interventions such as physical exercise.

KEYWORDS

Stroke; Tissue plasminogen activator; Thrombolysis; Hemorrhage; Neutrophils; Matrix metalloproteinases

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ABBREVIATIONS

AT1R: Angiotensin-2 Type 1 Receptor

BBB: Blood Brain Barrier

ECM: Extra Cellular Matrix

GCSF: Granulocyte Colony Stimulating Factor

HDL: High Density Lipoprotein

HT: Hemorrhagic Transformation

ICAM-1: InterCellular Adhesion Molecule 1

MAC-1: Macrophage-1 antigen

MCAO: Middle Cerebral Artery Occlusion

MCP-1: Monocyte Chemoattractant Protein-1

MMP: Matrix MetalloProteinase

NET: Neutrophil Extracellular Trap

NIHSS: National Institutes of Health Stroke Score

NLR: Neutrophil – Lymphocyte Ratio

NURR1: Nuclear Receptor Related-1 protein

PARP: Poly-ADP Ribose Transferase

PDE3: Phospho-Di-Esterase 3

PMN: PolyMorphoNuclear cell

PPAR: Peroxisome Proliferator-Activated Receptor

ROS: Reactive Oxygen Species

TIMP: Tissue Inhibitor of MMP

TNF α : Tumor Necrosis Factor alpha

tPA: tissue Plasminogen Activator

VAP-1: Vascular Adhesion Protein-1

VCAM: Vascular Cell Adhesion Molecule

1. Introduction

Stroke is, after cardiovascular diseases and cancer, one of the major public health problems in industrialized countries. In 2013, about 6.4 million deaths were linked to stroke worldwide (Kim et al., 2015). Stroke is the third cause of mortality, the second cause of dementia and the first cause of disability in adults. The main therapeutic strategy is the rapid restoration of blood flow in the ischemic area, which favors long-term recovery and helps to limit cognitive consequences (Powers et al., 2018). Pharmacologically, the principle of treatment is based on the acceleration and amplification of physiological fibrinolysis - i.e. the degradation of thrombus fibrin by plasmin - in order to limit the severity and duration of ischemia. The only authorized agent is recombinant tissue Plasminogen Activator (tPA, or alteplase), homologous to endogenous human tPA and sharing similar fibrinolytic properties.

Fibrinolysis (or thrombolysis) is beneficial if administered in the first few hours after the onset of cerebral ischemia under very specific conditions (Wardlaw et al., 2014). Beyond the effects at the acute phase, thrombolysis also limits disability with decreased levels of dependency at 3 months. There are two major limitations restricting its use and efficacy. The first limitation stems from the risk of symptomatic cerebral hemorrhage. Hemorrhagic transformations (HT) are a well-known complication of stroke itself, but their frequency is increased when tPA is administered. Symptomatic cerebral hemorrhages were observed in the first 36 hours after stroke in 6.4% of patients treated with tPA, but only in 0.6% of patients who received a placebo (NINDS rt-PA Stroke Study Group, 1995). The mortality rate in these patients is very high, with some studies reporting death rates up to 60% in patients who had developed intracranial hemorrhage (Gore et al., 1995). A meta-analysis of 27 clinical trials comparing cerebral fibrinolysis with different types of thrombolytic agents (10,187 patients) confirmed excess mortality at 3 months in patients who had received thrombolysis, due to a marked increase in the risk of symptomatic cerebral hemorrhage (Wardlaw et al., 2014). The risk for tPA-induced hemorrhages is time-dependent and accounts for its narrow therapeutic window.

Treatment is deemed beneficial up to 4h 30 min after symptom onset, the level of risk becoming excessive after 6 hours. As a consequence, tPA is used to treat approximately only 10% of the eligible patients (Fugate & Rabinstein, 2014).

The second limitation is related to the resistance of the thrombus to tPA-induced lysis. The mechanisms of resistance to thrombolysis remain largely unknown. The analysis of thrombi gathered from stroke patients revealed that the characteristics of the thrombi played an important part in the response to tPA. The physical properties (shape, length, porosity) influence clot exposure to tPA and treatment efficacy (De Meyer et al., 2017). The composition of the clot, especially the red blood cell content, has also been linked to variable recanalization rates (Hashimoto et al., 2016). Finally, the overall effect of tPA could be altered if thrombus regeneration occurs through cellular inflammatory pathways, as the concept of thrombo-inflammation suggests (Tsvigoulis et al., 2018). This lack of recanalization drives the development of mechanical thrombolysis, i.e. thrombectomy. Several clinical trials recently demonstrated the efficacy of this therapeutic approach in combination with pharmacological thrombolysis, as confirmed by recent meta-analyses (Caranfa et al., 2018; Mistry et al., 2017; Tsvigoulis et al., 2018).

In this context of thrombolysis, thrombo-inflammation and HT, a role for polymorphonuclear cells (PMNs or neutrophils) was proposed (De Meyer et al., 2016; Ruhnau et al., 2017). Neutrophils are recognized as key players in thrombo-inflammation at the acute phase of stroke (Price et al., 2004). They are one of the main sources of released molecules (pro-inflammatory cytokines, matrix metalloproteinases) that contribute to the breakdown of the neurovascular unit (Iadecola & Anrather, 2011; Jin et al., 2010; Moskowitz et al., 2010). In addition, tPA is able to act directly on neutrophils to modify their level of activation and release activity (Carbone et al., 2015; Cuadrado et al., 2008).

The aim of this review is to assess and clarify the role of neutrophils in tPA-induced hemorrhages, and their potential as biomarkers and therapeutic targets. To do so, we conducted a comprehensive

search on Medline, Pubmed and Embase for studies published until April 30th, 2018. The keywords used were stroke, cerebral ischemia, thrombolysis, tissue plasminogen activator, alteplase, hemorrhagic transformation, neutrophil, leukocyte, leukocyte-endothelium interactions, inflammation, and oxidative stress.

2. Neutrophils in the physiopathology of stroke

Discussing the role of neutrophils in thrombolysis requires beginning with a review of their role during stroke. In stroke, tissue damage and resulting neurological deficits originate from the implementation of various pathways, deleterious and protective. The vascular occlusion primes a complex sequence of events involving excitotoxicity, oxidative stress, inflammation (Iadecola & Anrather, 2011; Khoshnam et al., 2017). Neutrophils appear as central players, as they directly and indirectly play a part and influence the course of these events, including the response to thrombolysis.

Neutrophils are the first circulating immune cells to be recruited after stroke onset. Within 15 minutes, neutrophils interact with activated endothelial cells. They slow down and roll at the surface of the endothelium through interactions of their surface integrin Macrophage-1 antigen (MAC-1) with endothelial P and E-selectins. This rolling phenomenon can be witnessed in pial vessels within 2 hours. Neutrophils then attach to the endothelium through interactions with adhesion molecules, especially InterCellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecule (VCAM). From here, they can extravasate, infiltrate the parenchyma and migrate toward the lesion following a gradient of chemokines and cytokines (Kim et al., 2016). In experimental stroke models, neutrophils can be detected in the microvascular compartment as early as 30 minutes after occlusion, reaching peak concentrations at 12 hours (Garcia et al., 1994). As for infiltrated neutrophils, they can be detected in the ischemic parenchyma 6 hours after stroke; their density is maximal 48 to 72 hours after stroke (Kim et al., 2016; Zhang et al., 1994). Post-mortem clinical data support these experimental data, evidencing ICAM-1 upregulation in blood vessels and neutrophil infiltration in the ischemic parenchyma (Lindsberg et al., 1996).

Given their phagocytic abilities, neutrophils are involved in the removal of cellular debris, mandatory for tissue regeneration. But they also play a direct part in tissue damage. Neutrophils have the capacity to synthesize and secrete pro-inflammatory mediators such as Tumor Necrosis

Factor alpha (TNF α), thus promoting leukocyte infiltration and participating in the establishment of a sustained inflammatory reaction. They are also partially responsible for the oxidative phenomena majored by reperfusion as they are equipped with oxidative enzymes for phagocytosis. With this sudden influx of oxygen, the enzyme NADPH-oxidase produces superoxide in excess. Superoxide itself and Reactive Oxygen Species (ROS) derived from it act as intercellular messengers and prompt the expression of endothelial adhesion molecules. This overabundance of ROS also causes the formation of peroxynitrite by combination with nitric oxide, thus amplifying the oxidative stress and leading to enzyme dysfunction and cellular damage (Khoshnam et al., 2017).

Whether attached to the endothelium or infiltrated in the parenchyma, activated neutrophils degranulate *in situ*. The released enzymes, including elastase, proteases, collagenases, degrade the surrounding structures: in particular the extracellular matrix (ECM), tight and adherens junctions constituting the Blood-Brain Barrier (BBB). One family of enzymes is of particular interest. The Matrix Metallo-Proteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases able to degrade all components of the extravascular matrix (laminin, collagen and fibronectin). Two major isoforms are involved in stroke: MMP-2 and MMP-9. MMP-9 is secreted as an inactive pro-MMP-9 that is rendered active after cleavage by many enzymes (MMP-2, MMP-3, plasmin, urokinase-type plasminogen activator, tPA). Numerous studies have documented the role of MMP-9 in the breakdown of the BBB and in the formation of vasogenic edema, thus worsening stroke outcome (Rosell et al., 2006).

Besides contributing to inflammation, oxidative stress and structural degradation, neutrophils yet participate in another process: the no-reflow phenomenon, resulting from vascular dysfunction at different levels (Granger & Kvietys, 2017). The rolling, adhesion and infiltration of neutrophils at the surface of cerebral veins and venules has been linked to post-ischemic endothelial dysfunction (Palomares & Cipolla, 2011; Pétrault et al., 2005), as well as the disruption of the ECM and cellular junctions. But neutrophils are also involved in the formation of thrombi occluding microvessels. They

release tissue factors and proteases activating coagulation factors, thus leading to fibrin accumulation and clot formation (Ruhnau et al., 2017). Attached neutrophils also offer an interface for platelets to adhere. They interact, via their surface integrins, with activated endothelial cells, the exposed ECM and platelets (De Meyer et al., 2016). Recently, it was shown that their contribution to the constitution and stabilization of new thrombi relies on Neutrophil Extracellular Traps (NETs) (Michel & Ho-Tin-Noé, 2015). NETs constituted of extracellular strands of DNA associated with histones and proteins normally contained in granules such as myeloperoxidase, elastase, and MMPs. NETs are part of the defense mechanisms and released to entrap pathogens. Their role is complex (Bonaventura et al., 2018), but they have been shown to be major constituents of cerebral thrombi retrieved from stroke patients (Laridan et al., 2017).

3. Neutrophil involvement in tPA-induced hemorrhages

Thrombolysis exacerbates the risk of HT after stroke and symptomatic HT is thought to affect up to 7% of patients (Yaghi et al., 2017). Their definition relies on clinical and radiological criteria: symptomatic or asymptomatic, location and type of bleeding: petechial or parenchymal. Most events happen within 36 hours (Yaghi et al., 2017). The pathophysiology of cerebral hemorrhage secondary to thrombolysis remains unclear and is still subject to discussion (Palomares & Cipolla, 2011; Yaghi et al., 2017). It appears to result from the combination of ischemia-reperfusion damages, in particular opening of the BBB and tPA pharmacological effects.

Experimental models have been developed in recent years to study the pathophysiological mechanisms of the hemorrhagic complications of thrombolysis with tPA. They are adapted from two well-established models of cerebral ischemia-reperfusion based on the temporary occlusion of the middle cerebral artery: Middle Cerebral Artery Occlusion (MCAO) models (Shearer et al., 2017). The occlusion can either be mechanical: introduction of an occluding filament, pulled out to allow reperfusion; or embolic: in situ injection of thrombin or of an autologous clot (Durukan & Tatlisumak, 2007). The principle is based on the administration, at different time points after the onset of cerebral ischemia, of tPA at various doses, bolus or continuous infusion. Rodent models are most frequently used and hemorrhagic complications, generally in the ischemic area, can be observed a few hours after thrombolysis. In terms of pathophysiology, the most accurate models are the thromboembolic ones (Aoki et al., 2002; Tejima et al., 2001). Post-reperfusion injections of tPA in mechanical MCAO models appeared not suitable to study hemorrhagic transformations, as the impact of clot thrombolysis is not being replicated (Aoki et al., 2002). Nevertheless, the injection of a solution resulting from the action of tPA on an autologous clot, replicating the phenomenon of thrombolysis *ex vivo*, got around this limitation and allowed to show that the clot itself and its breakdown by tPA play an essential role in the occurrence of post-thrombolysis hemorrhage (Gautier et al., 2003; Kahles et al., 2005).

The risk of thrombolysis hemorrhages increases with the duration of occlusion, reperfusion and vascular risk factors (Jickling et al., 2014). Reperfusion restores blood flow in injured vessels, extravasation ensuing because of the damaged BBB. As tPA accelerates reperfusion, it inevitably increases the risk of HT. Other tPA-related factors favoring HT are the coagulopathy linked to hypofibrinogenemia that can last up to 24 hours after tPA treatment and direct blood vessel injury promoted by tPA (Kastrup et al., 2008; Yaghi et al., 2017). A dynamic MRI study showed that after ischemia, the vascular dysfunction depended on the timing of tPA administration. Hemorrhages were increasingly numerous as the administration of tPA was delayed, owing to a greater impact on BBB permeability (Zhang et al., 2014). A contributing role for neutrophils was proposed given their unique influence on the neuroglial unit during cerebral ischemia, and their ability to interact with tPA (del Zoppo, 2009; Roever & Levine, 2015) (figure 1).

Neutrophils directly contribute to the development of HT by promoting ischemic damages and partaking in the disruption of the BBB. Adherent neutrophils are early contributors to BBB disruption as they release MMPs among other products at the surface of blood vessels, and later on infiltrated neutrophils do the same in the parenchyma (Justicia et al., 2003). A few hours after experimental ischemic stroke, MMP-9 levels significantly increased; this phenomenon was aggravated and hastened in combination with thrombolysis with tPA (Lenglet et al., 2014). MMPs are thought to contribute to cerebral vascular impairment and tPA-related hemorrhages (Sumii & Lo, 2002; Tsuji et al., 2005; Turner & Sharp, 2016). The occurrence of post-thrombolysis hemorrhages was coincidental with an increase in cerebral vascular permeability and MMP-9 upregulation (Kahles et al., 2005). tPA increases in a dose-dependent manner the activities of plasma MMP-2 and MMP-9 (Golab et al., 2015) and induced MMP expression by endothelial cells (MMP-3) and astrocytes (MMP-2) (Jickling et al., 2014). The detrimental role of MMP-9 during thrombolysis was recently corroborated as the plasma variations of MMP-9 were independently associated with death or symptomatic intracerebral hemorrhage (Inzitari et al., 2013). In addition, a clinical study on 327 stroke patients treated with tPA

demonstrated that the MMP-9/Tissue Inhibitors of MetalloProteinases (TIMPs) ratios were predictors of intracerebral hemorrhages when elevated (Piccardi et al., 2015).

There are interactions taking place between neutrophils and tPA augmenting the risk of HT even more. tPA was responsible for an elevation in adherent and infiltrated neutrophils in an ischemia-reperfusion mouse model (Uhl et al., 2014). The pivotal interaction seems to be the triggering of neutrophil degranulation by tPA. This phenomenon, and the subsequent functional and structural degradation of the vascular compartment, was corroborated by several clinical studies. The analysis of brain and plasma samples confirmed that this phenomenon was accounting, at least partly, for the occurrence of post-thrombolysis hemorrhages (Carbone et al., 2015; Cuadrado et al., 2008; Rosell et al., 2008). A peak concentration of MMP-9 coming from neutrophil degranulation was detected in the first hours following the infusion of tPA in plasma collected from stroke patients who underwent thrombolysis (Carbone et al., 2015).

The role of neutrophils in thrombolysis hemorrhages was supported by experimental pharmacological modulation assays. Vinblastine-induced leuconeutropenia counteracted the tPA-induced massive infiltration of neutrophils – as well as the stroke-induced PMN infiltration - in the ischemic area. It also prevented tPA-induced hemorrhages and improved prognosis (Gautier et al., 2009; Pétrault et al., 2005). Accordingly, neutropenia induced with an antibody specifically directed against neutrophils (mAbRP-3) just before stroke and thrombolysis prevented tPA-induced hemorrhages (Gautier et al., 2009). Inducing neutrophilia with Granulocyte Colony Stimulating Factor (GCSF) gave conflicting results. Whereas GCSF appeared clearly neuroprotective in experimental stroke models through vasculo- and angiogenesis mechanisms, the combination with tPA seems to exert contrasting effects depending on the experimental setting (Bråtane et al., 2009; Minnerup et al., 2008; Sevimli et al., 2009; Sobrino et al., 2010; Solaroglu et al., 2015; Strecker et al., 2010). GCSF appeared to attenuate post-thrombolysis hemorrhages through vascular protection whereas neutrophilia elicited them through an increased release of MMP-9 from neutrophils (dela Peña et al.,

2015; Gautier et al., 2014). In a specific model of neonatal hypoxia ischemia, the beneficial neuroprotective effect of GCSF was observed only if neutrophilia was prevented (Doycheva et al., 2014).

Of course, HT do not result from the actions of neutrophils alone, and likely originate from the combined effects of several factors. Other early contributors are endothelial cells and pericytes, followed as the ischemic injury develops by neuron, astrocyte and microglia activation (Jickling et al., 2014). Oxidative stress plays a major part in HT, as does neuroinflammation (Wang et al., 2015). Another set of factors also plays a part in the occurrence and severity of HT: Vascular risk factors and clinical features at the acute phase of stroke, such as age, hypertension/blood pressure, diabetes/hyperglycemia, and the use of concomitant drugs are involved and their influence should not be underestimated (Lansberg et al., 2007; Larrue et al., 2001). Usually not or only partly replicated in preclinical studies, they are on the other hand easily assessable in the clinical setting (Menon et al., 2012).

4. The neutrophil as a biomarker of tPA-induced hemorrhages

The involvement of neutrophils in tPA-induced hemorrhages naturally led to consider them as potential biomarkers for HTs. Data already point towards a role for circulating neutrophils in stroke prognosis (Foerch et al., 2009). Pagram and collaborators found a strong correlation between circulating neutrophil counts and ischemic core volumes measured by MRI (Pagram et al., 2016). The Caliber study, reviewing 7,881 events, found an increased risk of stroke in patients with a high neutrophil count: HR=1.36 [95% CI 1.17 – 1.57] (Shah et al., 2017). Low neutrophil counts were associated with lower National Health Institutes Stroke Scores (NIHSS) and lesser risk for in-hospital mortality (Fang et al., 2017). Patients with a high neutrophil count after a minor or transient ischemic stroke were at higher risk of stroke recurrence in the 90 days following the first event (Zhu et al., 2018). In terms of hemorrhagic risk, white blood cell counts were significantly increased in the HT group after intra-arterial thrombolysis: OR=1.097 [95% CI 1.012 – 1.190] (Xing et al., 2014). An increase in neutrophil counts after tPA administration and a high neutrophil count 24 hours after stroke onset were predictive of an increased risk for death or major disability within 3 months (Shi et al., 2018).

Clinical studies also identified the Neutrophil – Lymphocyte Ratio (NLR) as a predictive marker of hemorrhagic transformations. A low NLR in the 24 hours following stroke onset was associated with a better outcome at 3 months (Pagram et al., 2016; Qun et al., 2017;) and a lesser risk of short-term mortality (Shi et al., 2018; Tokgoz et al., 2013). Patients with NLR levels above 4.80 had a 3.71-fold increased risk for symptomatic intracerebral hemorrhages: adjusted OR=3.71 [95% CI 1.97 – 6.98]. These parameters were also associated with a higher mortality and a poorer functional outcome 3 months after stroke (Maestrini et al., 2015). In another study, blood samples were collected over 48 hours after tPA treatment in 189 Chinese stroke patients. A high NLR was associated with a high risk of hemorrhagic transformations: adjusted OR=1.14 [95% CI 1.05 – 1.23], and patients with NLR levels above 10.59 had an 8-fold increased risk for symptomatic intracerebral hemorrhages: adjusted OR=7.93 [95% CI 2.25 – 27.99] (Guo et al., 2016).

Neutrophils were identified as a main source of MMP-9 in areas of hemorrhage (Turner & Sharp, 2016). As MMP-9 is also expressed in neutrophils in peripheral blood (Tang et al., 2006), MMP-9 levels could be used as predictors of intracerebral hemorrhage in tPA-treated patients (Castellanos et al., 2007). In fact, high baseline plasma levels of MMP-9 were predictive of late hemorrhagic events (Montaner et al., 2003). In addition, NETs and their constituents appear as interesting biomarkers of the neutrophil activation and potentially useful in anticipating the response to tPA and stroke prognosis (Michel & Ho-Tin-Noé, 2015). NET constituents are easily measurable in plasma and tissue: citrullinated histone-3, nucleosomes, cell-free DNA, elastase and myeloperoxidase are among the most commonly used. These markers can be used to track neutrophil recruitment and activation in experimental ischemic stroke (Perez-de-Puig et al., 2015). In a cohort of stroke patients, plasma levels of NET markers were positively correlated with stroke severity and 1-year risk of death (Vallés et al., 2017). Furthermore, NETs seem to play an important part in the constitution of thrombi in ischemic stroke. Two studies have shown that NETs are an important part of their structure as constituents of the backbone on which cells and proteins can adhere. Their presence was associated with thrombolysis resistance, as tPA was less efficient on thrombi with a rich extracellular DNA and neutrophil content (Ducroux et al., 2018; Laridan et al., 2017). Further studies are required to assess the potential interactions between NETs and tPA, as data suggest that tPA could alter neutrophil anti-infectious properties and NET release in stroke patients (Vogelgesang et al., 2017).

Others markers could also be useful (Lu et al., 2018). For example, tPA regulated the pro-inflammatory transcription factor Nuclear Receptor Related-1 protein (NURR1). This transcription factor, whose expression increases after thrombolysis, causes hemorrhagic injury in the rat, and patients with post-thrombolysis hemorrhages were found to have higher basal levels of this factor than those without hemorrhages (Merino-Zamorano et al., 2015). Likewise, the Vascular Adhesion Protein-1 (VAP-1) is involved in recruitment of neutrophils. Measuring its activity before tPA administration was demonstrated to be predictive of tPA-induced hemorrhages (Hernandez-Guillamon et al., 2010).

5. Reducing hemorrhagic transformations by neutrophil targeting

Reducing the risk of hemorrhages after thrombolysis could be a strategy to extend the otherwise limited therapeutic window of tPA, and may even pave the way for a broader use of tPA. Many pharmacological approaches have been tested with the aim of reducing the impact of stroke and hemorrhagic complications in general (Zhang et al., 2012). As evident contributor to tPA hemorrhages, neutrophils could be valuable pharmacological targets. Strategies targeting neutrophils directly are limited, as discussed above, by difficulties to translate their use to the clinical setting (induced neutropenia, anti-neutrophil antibodies) or to demonstrate a clinical benefit (MMP-9 inhibitors for example). Conversely, indirect interventions targeting pathophysiological pathways involved in the recruitment and activation of neutrophils appear more valuable (figure 2).

- ***Statins***

The neurovasculoprotective potential of statins in ischemic stroke is well-established in experimental models as preventive or acute phase treatments (Ouk, et al., 2014a; Potey et al., 2015). This benefit mainly relies on their anti-inflammatory effects, decreased neutrophil adhesion and infiltration, preservation of vascular properties (function and structure) and reduced microglial activity (Potey et al., 2015; Wang et al., 2006; Zhang et al., 2005). These effects rely at least partly on the modulation of the nuclear receptor Peroxisome Proliferator-Activated Receptor (PPAR)-alpha and are independent of their lipid-lowering effects (Miedema et al., 2012; Nardi et al., 2011, 2012; Rocco et al., 2012).

In combination with tPA, data are controversial. In vitro experiments showed that lovastatin, even at high doses, did not have an impact on tPA thrombolytic activity (Kandadai et al., 2012). When hypertensive rats received simvastatin 15 minutes after the induction of ischemia and tPA 3 hours later, no difference in terms of neuroprotection, MMP-2 and MMP-9 activities and rate and severity of hemorrhages compared to the placebo group treated with tPA only were found (Campos et al., 2013). In a thromboembolic model, atorvastatin administered before or concurrently to thrombolysis decreased neutrophil adhesion and infiltration, reduced the expression of MMP-9 and the risk of

hemorrhage (Liu et al., 2006; Zhang et al., 2009). On the other hand, a preventive treatment with simvastatin increased significantly the incidence and volumes of tPA-induced hemorrhages in rabbits submitted to embolic cerebral ischemia (Lapchak & Han, 2009).

The potential benefits of statins in primary and secondary stroke prevention have been evaluated in several clinical studies (Amarenco et al., 2006; Phipps et al., 2013; Taylor et al., 2013). Some studies have shown that the preventive use of a statin (before stroke) was associated with a better neurological and improved functional prognosis in thrombolized patients (Alvarez-Sabín et al., 2007; Engelter et al., 2011) whereas others failed to show any benefit or deleterious effect (Campos et al., 2013; Martinez-Ramirez et al., 2012; Meier et al., 2009; Miedema et al., 2010; Tsivgoulis et al., 2015). Among these studies, even if an association was found between previous statin use and hemorrhagic transformations, the 3-month prognosis was not altered (Martinez-Ramirez et al., 2012; Meier et al., 2009). Scheitz and collaborators (Scheitz et al., 2013; 2014) reported a long-term beneficial effect of statins yet found an increased and dose-dependent risk of thrombolytic hemorrhage associated with previous statin treatment. The underlying mechanisms remain largely unknown. Zhao and Zhang reported that a previous statin treatment had no impact on MMP-9 levels in thrombolized patients at 12, 24 and 72 hours, with no change of the hemorrhagic risk or of the patients' course (Zhao & Zhang, 2014).

Data regarding the initiation of a statin treatment concomitantly to tPA at the acute phase of stroke is controversial. In some studies, there was a tendency towards improvement or a significant neurological and functional improvement (Cappellari et al., 2013; Geng et al., 2016; Tong et al., 2015), and a lower risk of mortality (Scheitz et al., 2016). The long-term benefit seemed to depend on the duration of the statin treatment, for at least three weeks after stroke (Tong et al., 2015). Others did not evidence a beneficial effect (Montaner et al., 2016), but overall, none raised safety concerns. The risk of hemorrhagic transformation was not increased in patients treated with the combination statin-tPA (Cappellari et al., 2013; Geng et al., 2016; Montaner et al., 2016; Scheitz et al., 2016). A relatively recent meta-analysis concluded that the use of statins at the acute phase was beneficial on

the course of stroke, benefit that was lost if patients underwent thrombolysis (Ni Chroinin et al., 2013).

- ***Fibrates***

Fibrates are agonists of the nuclear receptor PPAR-alpha. The activation of PPAR-alpha at the acute phase of experimental ischemia, or a few days later as a secondary stroke prevention strategy, confirmed the value of fenofibrate, irrespectively of the treatment schedule, as a protector of the neuroglivascular unit as a whole (Deplanque et al., 2003; Ouk et al., 2009; 2014b). This protection was exerted through the modulation of the interactions between neutrophils, the vascular wall and microglia (decreased adhesion and rolling of neutrophils, decreased parenchymal and vascular inflammation, reduction in vascular dysfunction and infarct volume in particular). This modulation was associated with a significant reduction in the hemorrhagic risk following tPA administration. A concomitant reduction in the vascular adhesion and infiltration of neutrophils and in microglial activation was shown to be beneficial (Gautier et al., 2015). Nevertheless, there is no available clinical data on PPAR alpha modulation combined with tPA, and whether such an association could reduce the risk of hemorrhagic transformations has yet to be evaluated. Likewise, it is not known if PPAR alpha modulators alter could tPA thrombolytic properties.

- ***Angiotensin II Receptor type 1 blockers (sartans)***

Angiotensin-2 Type 1 Receptor (AT1R) blockers, or sartans, exert beneficial effects in ischemic stroke via pharmacological mechanisms distinct from their on-label indication, hypertension. Sartans have been shown to modulate various pathophysiological pathways in experimental stroke, especially inflammation. Treatment with an AT1R blocker decreased the production of pro-inflammatory cytokines (TNF α , interleukin-1 β), chemo-attracting factors such as Monocyte Chemoattractant Protein-1 (MCP-1), adhesion molecules (ICAM-1, P-selectin), and MMPs (Hosomi et al., 2005; Iwanami et al., 2010; Kasahara et al., 2010; Kozak et al., 2009).

The documentation of potential effects of sartans on neutrophils in stroke is scarce and a clinical effect has yet to be proved. Angiotensin-2 stimulates the expression of MMPs. It was shown

that telmisartan, both an AT1R blocker and a PPAR gamma activator, just after ischemia was neuroprotective in a MCAO model, beyond its effect on blood pressure and through the reduction of MMP-9 and of the inflammatory response (Kono et al., 2015). Candesartan applied 3 hours after embolic MCAO was neuroprotective and limited the incidence of secondary hemorrhages after delayed thrombolysis (tPA applied 6 hours after stroke onset). MMP-9 activity was increased, and MMP-3 activity decreased (Ishrat et al., 2013).

- ***Thiazolidine-diones***

Hyperglycemia is associated with reperfusion injury and promotes inflammation and oxidative stress. Acute hyperglycemia is associated with a poor prognosis in patients with ischemic stroke, and is a well-known risk factor of secondary hemorrhagic transformations (Ahmed et al., 2010; Poppe et al., 2009). The value of glycemia on admission is decisive and has a major influence on stroke outcome, tPA efficacy and risk of hemorrhagic transformations, whereas the history of glycemic control is of little value (Litke et al., 2015).

Amongst the available anti-diabetic drugs, the thiazolidine-dione class is of particular interest in stroke. They are agonists of the nuclear receptor PPAR gamma. They modulate glycemia and multiple other pathways that grant them neuroprotective properties. Rosiglitazone limited the recruitment and extravasation of neutrophils to the ischemic area when administered preventively (Luo et al., 2006) or following experimental stroke (Tureyen et al., 2006). It was still protective and prevented neutrophilia 24 hours after MCAO (Allahtavakoli et al., 2009).

Rosiglitazone also proved to be beneficial when used as a combined treatment with tPA in a rat model of embolic stroke. It potentiated the protective effects of tPA, and increased the time window for safe tPA administration. In the ischemic area, MMP-9 enzymatic activity was reduced, and blood vessel structure preserved (Wang et al., 2009). It was also shown to reduce the occurrence of secondary hemorrhagic transformations induced by acute hyperglycemia in rats submitted to MCAO (Zhang et al., 2013). Clinical trials are needed to assess if these beneficial effects could be

translated to humans. It would also be interesting to evaluate if PPAR gamma modulators modify the thrombolytic efficacy of tPA.

- **Free radical scavengers**

Free radical scavengers were proposed as a joint therapy to tPA thrombolysis to counteract the rebound in oxidative stress that accompanies reperfusion. The main candidate NXY-059 was able to reduce the incidence of hemorrhagic transformations in studies based on a rabbit thromboembolic stroke model (Lapchak et al., 2002a; 2002b; Lapchak et al., 2004). These results could not be replicated in patients. The SAINT-I and SAINT-II clinical trials combining tPA with NXY-059 failed to show beneficial effects, and the incidence of hemorrhagic transformations was not reduced either (Diener et al., 2008).

Edaravone by itself is a neuroprotectant authorized in Japan as an acute stroke treatment. When associated with tPA, edaravone amplified the thrombolytic efficacy of the treatment and provided more protection than tPA alone. The neuroprotective effects are various but notably involve a downregulation of MMPs, thus protecting the BBB (Kikuchi et al., 2017; Sun et al., 2014). Whether edaravone was administered during occlusion or concomitantly to tPA, the risk of hemorrhages was decreased, as were the expression and activity of MMPs (Yagi et al., 2009; Yamashita et al., 2009). It is not known if this effect on MMPs is related to an effect on neutrophils. Edaravone has been shown to diminish the production of ROS, especially of superoxide, in neutrophils harvested from patients at the acute phase of stroke (Aizawa et al., 2006). There is no available data on whether edaravone could be able to modulate neutrophil pro-inflammatory pathways. The synergistic effects of the edaravone – tPA combination were confirmed *in vitro* on plasma from stroke patients (Kikuchi et al., 2017). So far, clinical trials have failed to confirm beneficial effects either on the efficacy of thrombolysis or on the reduction of hemorrhagic complications (Aoki et al., 2017; Tsuruoka et al., 2014).

Another anti-oxidant, resveratrol, was also tested in combination with thrombolysis. The outcome of patients receiving the combined treatment was significantly better regardless of whether

they benefited from early – less than 2 hours after stroke onset – or delayed – 2 to 4 hours – thrombolysis. In this group of patients, the plasma levels of MMP-2 and MMP-9 were significantly decreased compared to patients in the tPA/placebo group (Chen et al., 2016).

- **Minocycline**

Minocycline, an antibiotic of the tetracycline family, has been shown to exert pleiotropic effects responsible for its neuroprotective properties in ischemic stroke, including marked anti-inflammatory effects. These effects are mediated, at least partly, by the inhibition of the enzyme Poly-ADP-Ribose Transferase (PARP). Several studies investigated the potential of a combined treatment associating minocycline and tPA. *In vitro*, there were no modifications of the fibrinolytic properties of tPA (Machado et al., 2009). Minocycline started before or simultaneously with tPA induced a reduction in the incidence and volume of hemorrhagic transformations in parallel with a global neuroprotective effect. The levels and enzymatic activities of MMP-2 and MMP-9, measured in plasma and brain samples, were decreased (Fan et al., 2013; Machado et al., 2009; Murata et al., 2008). Fewer neutrophils were found infiltrated in the peri-infarct areas 16 hours after embolic MCAO and treatment with tPA and minocycline (Fan et al., 2013).

The clinical evaluation of minocycline in stroke is extensive, alone as an acute phase neuroprotectant or combined with tPA. The MINOS pilot study assessed the impact of minocycline started within 6 hours of stroke onset in 60 patients treated for 3 days. Minocycline was well-tolerated and no severe hemorrhagic transformation was reported, even in the 44 patients treated with tPA (Fagan et al., 2010). In this subgroup of patients, the levels of plasma MMP-9 were significantly reduced compared to patients treated with tPA alone (Switzer et al., 2011). The ongoing Australian study WAIMATSS (Blacker et al., 2013) was designed to specifically evaluate if an acute treatment with intravenous minocycline (started within 6 hours of symptoms onset, one dose every 12 hours for 3 days) could reduce the incidence of tPA-induced hemorrhages. The preliminary results did not show any difference in the rates of hemorrhagic transformations (Blacker et al., 2015)

- ***Cilostazol***

In vitro, the Phospho-Di-Esterase 3 (PDE3) inhibitor cilostazol decreased the expression of endothelial adhesion molecules and subsequently neutrophil adhesion (Omi et al., 2004). Its neuroprotective effects are well established in ischemic stroke and involve a reduction of neutrophil infiltration in the ischemic area when administered at the acute phase of stroke (Lee et al., 2007; Park et al., 2007). Whether used as a preventive or as an acute phase treatment, cilostazol also exerts protective effects and protects against tPA-related hemorrhages. Under both treatment conditions MMP-9 activity was reduced (Hase et al., 2012; Ishiguro et al., 2010; Kasahara et al., 2012).

As experimental data showed a neuroprotective effect in stroke, several clinical studies have been led evaluating this drug as an acute stroke treatment. Cilostazol tended to protect against complications and improve the functional prognosis at 3 months (Matsumoto et al., 2011; Nakamura et al., 2013). To date, the potential benefits of associating cilostazol to tPA have not been assessed.

- ***HDL***

Besides the well-established correlation between high plasma levels of High Density Lipoproteins (HDL) and low cardiovascular risk, HDL exerts an anti-inflammatory effect *in vivo* and inhibits neutrophil adhesion and recruitment in a model of hemorrhagic shock (Cockerill et al., 2001). Similar effects were evidenced in rats subjected to embolic MCAO. HDL administered 3 or 5 hours after occlusion helped to preserve the BBB against the ischemic injury, and inhibited neutrophil adhesion, recruitment and activation (Lapergue et al., 2010). When tPA was co-administered, the acute HDL treatment was still beneficial and modulated the same mechanisms. The rate of hemorrhagic transformations was significantly lower in animals treated with HDL combined with tPA (Lapergue et al., 2013). Interestingly, HDL do not alter the proteolytic activity of tPA and could be administered concurrently without modifying its thrombolytic efficacy (Lapergue et al., 2013). Clinical trials are needed to confirm the relevance of such a combination in ischemic stroke.

- ***Non pharmacological interventions: physical activity***

The beneficial influence of physical activity on outcome and recovery after ischemic stroke is linked to an upregulation of cerebral plasticity enhancing tissue repair and regeneration, and involves the activation of PPAR delta. Pre-stroke physical activity was linked to neuroprotection, via the promotion of anti-inflammatory pathways (Chrysohoou et al., 2005). It was shown to be responsible for a decrease in the vascular expression of ICAM-1, and in leukocyte infiltration in the ischemic parenchyma. A reduction in the expression of MMP-9 has also been reported. A similar effect on MMP-9 has been reported with post-stroke physical training (Pin-Barre & Laurin, 2015). Even if the benefits of physical activity in terms of recovery were not confirmed in a cohort of tPA-treated stroke patients (Decourcelle et al., 2015), there is no available clinical data on the impact on the safety profile of tPA. Whether the above-mentioned effects, evidenced experimentally, translate to a protective effect in the clinical setting towards tPA-induced hemorrhages remains unknown, and further studies are required.

6. Conclusion

Neutrophils are clearly accomplices in the occurrence of post-thrombolysis hemorrhages in stroke, primarily due to vascular alterations related to BBB disruption. Even if thrombectomy appears as a promising therapeutic option, tPA is still valuable despite the inherent risks. The next step is now to discover biomarkers and/or pharmacological agents that could be used to limit this risk in the clinical setting. Several reviews have addressed the matter of pharmacological modulation in this context (Jickling et al., 2015; Kanazawa et al., 2017), a recent one raising the question of the relevance of lipid-lowering drugs (statins and fibrates) as disease modifiers (medications that can have an overall influence on the course of the ischemic disorder and of thrombolysis) (Ouk et al., 2013). The latest recommendations rely on the reversion of the coagulopathy, the prevention of the extension of the hematoma by a close control of blood pressure and neurosurgical procedures (Yaghi et al., 2017). Pharmacological candidates targeting neutrophils require a thorough preclinical and clinical evaluation to confirm their relevance in the field of post-thrombolysis hemorrhages in ischemic stroke.

7. Conflict of Interest Statement:

"The authors declare that there are no conflicts of interest."

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Figure legends

Figure 1. Interplay between neutrophils, the neurogliovascular unit and the thrombolytic treatment at the acute phase of stroke: setting the stage for hemorrhagic transformation. ICAM-1, InterCellular Adhesion Molecule 1; MMP-9, Matrix MetalloProteinase 9

Figure 2. Pharmacological interventions targeting the neutrophil as a key actor in the development of post-stroke tissue damage and hemorrhagic complications secondary to tPA treatment. AT1R, Angiotensin-2 Type 1 Receptor; mAbs, monoclonal antibodies, MMPs, Matrix Metalloproteinases, PARP, Poly-ADP Ribose Transferase; PDE3, Phospho-Di-Esterase 3; PPAR, Peroxisome Proliferator-Activated Receptor; TZD, Thiazolidinediones

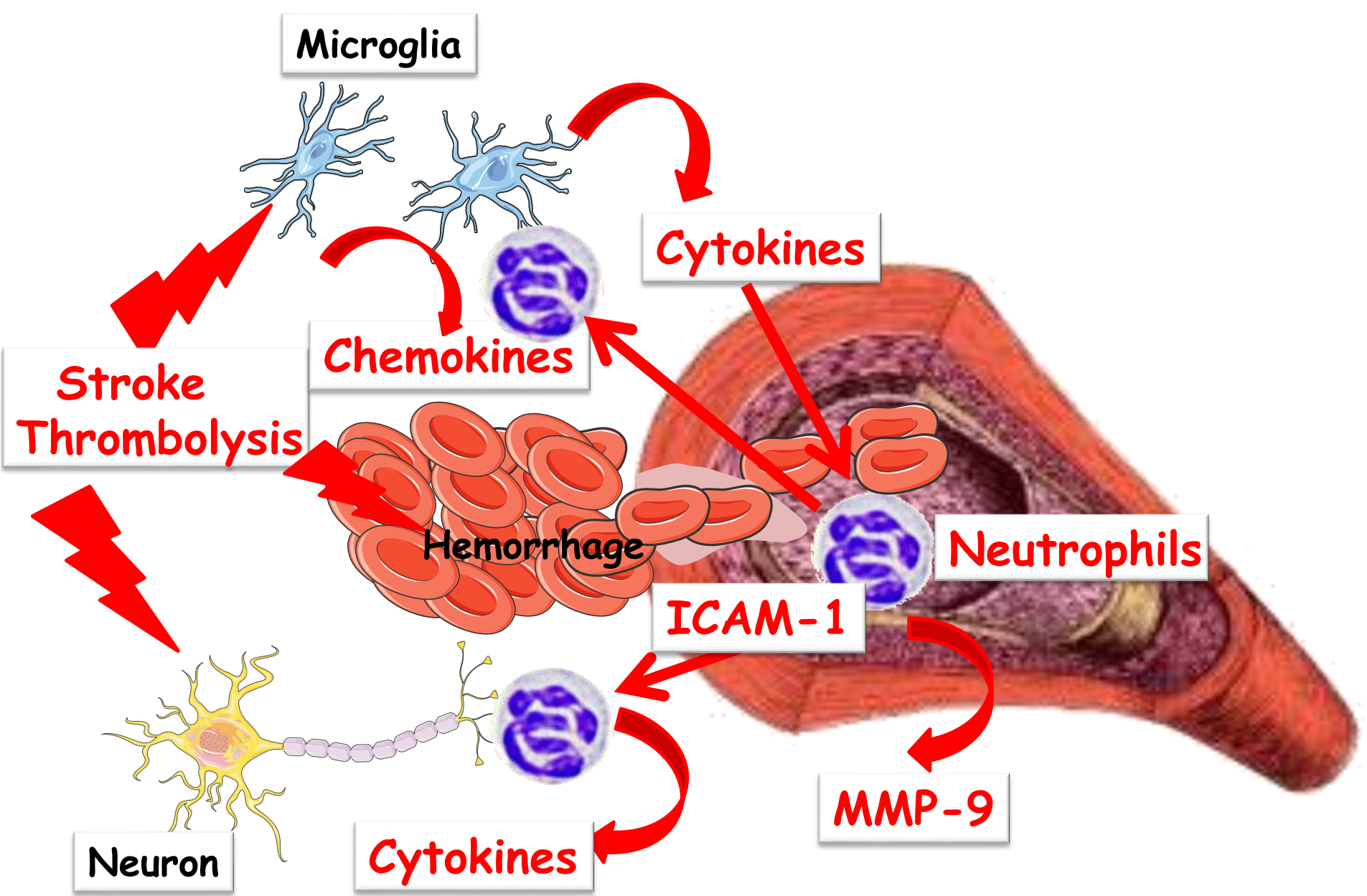


Figure 1. Interplay between neutrophils, the neurogliovascular unit and the thrombolytic treatment at the acute phase of stroke: setting the stage for hemorrhagic transformation. ICAM-1: InterCellular Adhesion Molecule 1; MMP-9: Matrix MetalloProteinase 9



Stroke

Direct interventions

Indirect interventions

mAbs
Pharmacological neutropenia
GCSF

Free radicals
Pro-inflammatory cytokines
Adhesion molecules

Free radical scavengers

Recruitment
Activation
Infiltration



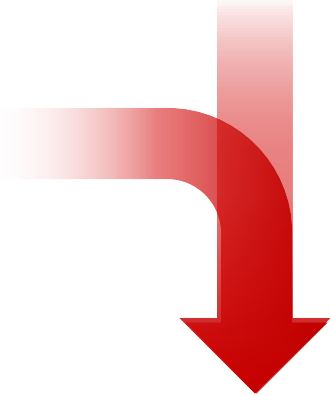
Neutrophil

Inflammation

Oxidative stress

Parenchymal damage

MMPs



Hemorrhagic complications

HDL

PARP

Minocycline

PPAR α

Fibrates

Statins

PPAR γ

TZD

AT1R

Sartans

PDE3

Cilostazol

PPAR δ

Physical activity

Figure 2. Pharmacological interventions targeting the neutrophil as a key actor in the development of post-stroke tissular damage and hemorrhagic complications secondary to tPA treatment mAbs: monoclonal antibodies, MMPs: Matrix Metalloproteinases, PARP: Poly-ADP Ribose Transferase, PPAR: Peroxisome Proliferator-Activated Receptor, AT1R: Angiotensin-2 Type 1 Receptor, TZD: Thiazolidinediones, PDE3: Phospho-Di-Esterase 3