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A large-scale histological investigation gives insight into the structure of ischemic stroke thrombi

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Worldwide, 15 million people suffer a stroke every year[1], it is therefore a significant global health and financial burden. In Europe alone, there were more than 600,000 people who endured a stroke in 2015 and this is estimated to increase to over 800,000 by 2035, equating to a massive financial burden of 45 billion Euros[2]. The majority of strokes (85%) are ischemic, triggered by a thrombus occluding a blood vessel, decreasing oxygen supply to the affected area and resulting in tissue damage. The remaining 15% of strokes are hemorrhagic and caused by rupture of a major vessel[3,4]. The severity of stroke can vary; in Europe it is the second most common cause of death[5] but even if the patient recovers, rehabilitation can take many months, or even years, and some individuals have to learn to live with permanent disabilities as a result of the stroke[2]. The best outcome for the patient is achieved if the blood flow is restored as promptly as possible[6]. European guidelines indicate that treatment for ischemic stroke should occur within the first 4.5 h of the onset of symptoms[3]. At present there are two approved treatment strategies for stroke. Most patients are treated with intravenous administration of recombinant tissue plasminogen activator (rtPA)[2], an enzyme that catalyses the conversion of plasminogen to plasmin, which induces thrombolysis (clot breakdown)[3]. A more recently employed technique is thrombectomy, where the clot is retrieved mechanically[7,8]. In many cases, these treatments are not sufficient to remove the occluding thrombus and restore blood flow. The underlying reason for this is still not fully understood. It is thought that this may be related to the great heterogeneity that is present in the stroke-causing thrombi that could make a clot more, or less, susceptible to degradation by rtPA or affect the likelihood of successful retrieval. Understanding the thrombus composition and structure could lead to more targeted and effective treatments for stroke[6].

The recent work of Staessens *et al.*[9] has made further advances in addressing the question of thrombus composition. In this paper the authors interrogated the structure of

177 thrombi from acute ischemic stroke patients isolated by thrombectomy and have made further steps towards deciphering the cellular components involved[9]. To our knowledge, this is the first study to use such a large number of samples to systematically characterize thrombus structure utilizing histological stains and immunofluorescence labelling for red blood cells (RBC), platelets, fibrin(ogen), von Willebrand factor (vWF) and DNA.

In the present study[9] thrombi were retrieved and analyzed from ischemic stroke patients, regardless of prior rtPA treatment. However, the authors did not correlate the results with the treatment to determine if there was any effect of the rtPA on thrombus structure. Further, they also acknowledge that findings may potentially be biased, as only retrievable thrombi were interrogated. In fact, there is evidence that RBC-rich thrombi are more easily removed by thrombectomy[10]. Nevertheless, all 177 thrombi in this study were subjected to the full range of analyses, with authors able to show that thrombi contain RBC-rich as well as platelet-rich areas. Regardless of the above-mentioned limitations, they elegantly demonstrate the continuum observed in the makeup of thrombi and the proportion of RBC-rich and platelet-rich areas, summarized in Figure 1. Within the different zones, Staessens *et al.*[9] showed that RBC-rich areas were composed of densely packed RBCs with a fine fibrin meshwork throughout. Nucleated cells, vWF and platelets were rarely seen in these areas. This relatively simple structure is in contrast to the platelet-rich areas where fibrin and vWF often colocalized, and leukocytes and an abundance of extracellular DNA were observed. The extracellular DNA was hypothesized to be the result of neutrophil extracellular traps (NETs) formation which has been shown to not only interact with platelets[11] but also to play a role in venous thrombosis[12,13]. However, further staining for citrullinated histone h3[14] and myeloperoxidase was not performed[15]. More specific staining for NETosis can confirm that the detected DNA is in fact neutrophil-derived and not induced by cell death or necrosis; additionally, it also makes it

possible to narrow down the specific pathway of the neutrophil activation and subsequent NET formation[16].

Putting these observations into the context of other studies the authors speculate that the rtPA resistance of platelet-rich thrombi could be due to DNA modification of the fibrin structure[17,18]. They also suggest that covalent cross-linking of vWF and fibrin by Factor XIII may contribute to the rtPA resistance[19-23]. Factor XIII is a transglutaminase which circulates in the blood as an inactive zymogen. Once activated by thrombin (to become FXIIIa), it can crosslink fibrin via isopeptide bonds which increases clot stability and makes it more resistant to fibrinolysis. FXIIIa has also been shown to crosslink vWF to fibrin[21]. As both fibrin and vWF have been observed to colocalise in the thrombus it would be interesting to probe the sections for Factor XIII to see if it was also present to support this hypothesis. This could explain why less than half of the patients that receive rtPA treatment to induce thrombolysis have a positive outcome[24,25]. The present work, therefore, strongly suggests DNAse and/or ADAMTS13 (a plasma metalloproteinase that cleaves vWF) may increase thrombolysis. These interesting findings warrant more in-depth study of rtPA alone or in combination with DNase1/ADAMTS13. In fact, Denorme *et al.*[26] have shown the presence of vWF in 36 ischemic stroke thrombi, but also that tPA treatment was more efficient after pre-infusion of ADAMTS13 in a murine stroke model[26]. Very recent work by Ahmed *et al.*[27] has shown that the platelet collagen and fibrin(ogen) receptor GPVI might be involved in stabilizing thrombi. Hence, an anti-GPVI agent could be used in combination with rtPA to promote disaggregation of platelet-rich thrombi[27]. This again provides further evidence that understanding thrombus structure could offer enhanced treatment strategies. It will be interesting to see whether the results of these mouse model and in vitro studies hold true in patient studies to see if such

combination therapies could be used in the future to increase the success of stroke treatment and patient outcomes.

Although the conclusions of Staessens *et al.*[9] are based on a large dataset, detailed quantification of the data obtained by immunohistochemistry for vWF, platelets, fibrin(ogen) and leukocytes is missing. Quantification of the impressive number of thrombi investigated could potentially provide not only numerical data in order to compare different thrombi, but also present the opportunity to interrogate the heterogeneity within an individual thrombus. A machine-learning approach to quantify labelled thrombus sections has been used previously[28,29] and the work by Staessens *et al.*[9] would benefit from a similar approach and therefore presents an opportunity for further exploitation of their large dataset.

This study is an important addition to a whole body of work that could significantly improve outcomes for stroke patients by improving treatment strategies. Similar studies state various patient parameters such as gender, age, preexisting conditions, stroke aetiology or even occlusion site[30,31]. Connecting them to thrombus morphology could be key in determining patient-specific therapeutic approaches. Therefore, follow-up studies should be pursued that investigate the correlation between thrombus structure and patient parameters, as well as plasma samples, to give more information on how different factors influence stroke severity, as well as patient outcome. Boeckh-Behrens *et al.*[32] investigated whether the underlying cause for ischemic stroke in 145 patients was due to a cardioembolic event. By combining semi-quantitative analysis of the thrombi with clinical data they concluded that most strokes were of cardioembolic origin[32]. More information about thrombus structure poses the potential to expand the choice and manner of treatment strategies thereby improving outcomes for ischemic stroke patients. In fact, there is evidence that in coronary thrombi fibrin content increases, while

the presence of platelets decreases the ischemic time (duration from onset of symptoms to the primary percutaneous intervention)[33].

Whilst this is a well-executed study, the conclusions are based upon observations and further experimental work is required. For example, laser capture microdissection could be utilized to not only visualize areas of interest, but also enable incorporation of other experimental measures such as proteomics, transcriptomics, or genomics to investigate regions/cells[34]. This type of approach would complement existing data obtained from mass spectrometry on stroke thrombi where Munoz *at al.*[35] identified 339 proteins commonly detected in the four patients investigated[35]. Further, as there is evidence that the composition of the thrombus plays an important role in the efficacy of thrombolytic treatment, it would be of interest to know what the underlying mechanism is for the formation of the two thrombi subpopulations (RBC- or platelet-rich). For example, different shear rates may influence the composition of the thrombus[36] and this could be something to further interrogate. Also, changes in the vessel wall, which can be picked up by noninvasive imaging techniques such as CT or MRI, have been correlated to an increased amount of RBC in the thrombus[37].

In summary, the authors are in the possession of a large treasure trove of samples. Other studies conducted on comparable specimens with similar methodologies did so with far fewer samples[38,39] or did not carry out such in-depth investigations[32]. The present study elegantly offers validation for textbook knowledge in a large cohort of patients, thereby laying the foundation for more in-depth investigations to improve treatment options for ischemic stroke patients.

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Conflicts of Interest

The authors declare no competing financial interests

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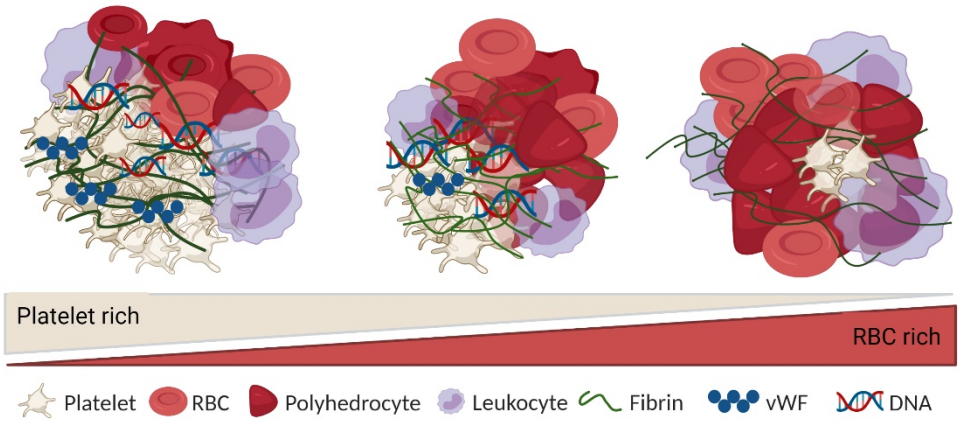

Figure 1 – Different thrombus morphology as observed by Staessens et al.[5]

Thrombi from stroke patients extracted by mechanical removal (thrombectomy) were found to be composed of a platelet-rich areas containing thick fibrin fibers, seemingly crosslinked with vWF and DNA. Red blood cell (RBC)-rich areas, with a loose fibrin network and some leukocytes were also present. Interestingly, leukocytes and DNA were shown to colocalize at the border between platelet- and RBC-rich areas. The 177 thrombi analyzed showed a full range of compositions from predominantly platelet-rich to almost entirely RBC-rich. This figure was created with BioRender.com.