Review

International Journal of Stroke WSO

International Journal of Stroke
0(0) 1–10

DOI: 10.1177/1747493019884527
journals.sagepub.com/home/wso
SAGE

Histological stroke clot analysis after thrombectomy: Technical aspects and recommendations

S Staessens[1], S Fitzgerald[2], T Andersson[3,4], F Clarençon[5], F Denorme[1], MJ Gounis[6], W Hacke[7], DS Liebeskind[8], I Szikora[9], ACGM van Es[10], W Brinjikji[11], KM Doyle[2] and SF De Meyer[1]; on behalf of the Clot Summit

Abstract

The recent advent of endovascular procedures has created the unique opportunity to collect and analyze thrombi removed from cerebral arteries, instigating a novel subfield in stroke research. Insights into thrombus characteristics and composition could play an important role in ongoing efforts to improve acute ischemic stroke therapy. An increasing number of centers are collecting stroke thrombi. This paper aims at providing guiding information on thrombus handling, procedures, and analysis in order to facilitate and standardize this emerging research field.

Keywords

Stroke, thrombus, thrombectomy, histology, clot

Received: 23 April 2019; accepted: 28 August 2019

Introduction

With a global prevalence of 25.7 million strokes per year, stroke is still one of the leading causes of death and sustained disability worldwide. The vast majority of strokes mainly are ischemic, caused by blood thrombi that occlude cerebral blood vessels.[1]

Since the publication of the "positive thrombectomy" trials in 2015, endovascular procedures have emerged to mechanically remove blood thrombi from the cerebral arteries.[2–6] This procedure has created the unique opportunity to provide stroke thrombus material for analysis. Studying thrombus pathology of retrieved material is crucial to improve our understanding, diagnosis, treatment, and secondary prevention of acute ischemic stroke.[7] The availability of thrombus material has sparked a novel subfield in stroke research with a rapidly increasing number of studies reporting valuable information using a wide range of techniques and sample sizes. A recent meta-analysis, however, concluded that lack of standardization may limit the generalizability of such studies.[8]

This paper aims to provide recommendations and guidelines on clot collection, processing, and analysis in order to facilitate and standardize this emerging research field (Table 1). Supplemental File 1 provides an overview of the most common methods and staining procedures that were used in previous studies.

Specimen retrieval

Mechanical thrombectomy procedures provide clot material from stroke patients. In general, histopathological analysis of stroke thrombi has been performed

[1]Laboratory for Thrombosis Research, KU Leuven Campus Kulak Kortrijk, Kortrijk, Belgium
[2]CÚRAM-Centre for Research in Medical Devices, National University of Ireland Galway, Galway, Ireland
[3]Department of Medical Imaging, AZ Groeninge, Kortrijk, Belgium
[4]Department of Neuroradiology, Karolinska University Hospital and Clinical Neuroscience, Karolinska Institute, Stockholm, Sweden
[5]Department of Neuroradiology, Pitié-Salpêtrière Hospital, APHP, Paris, France
[6]Division of Neuroimaging and Intervention and New England Center for Stroke Research, Department of Radiology, University of Massachusetts Medical School, Worcester, MA, USA
[7]Department of Neurology, University Hospital Heidelberg, Ruprecht-Karls University, Heidelberg, Germany
[8]Neurovascular Imaging Research Core and UCLA Stroke Center, University of California, Los Angeles, CA, USA
[9]Department of Neuroradiology, National Institute of Neurosciences, Budapest, Hungary
[10]Department of Radiology and Nuclear Medicine, Erasmus MC, University Medical Center (EMC), Rotterdam, The Netherlands
[11]Department of Radiology, Mayo Clinic, Rochester, MN, USA

Corresponding author:
SF De Meyer, Laboratory for Thrombosis Research, KU Leuven Campus Kulak Kortrijk, E. Sabbelaan 53, Kortrijk 8500, Belgium.
Email: simon.demeyer@kuleuven.be

Table 1. Specific recommendations

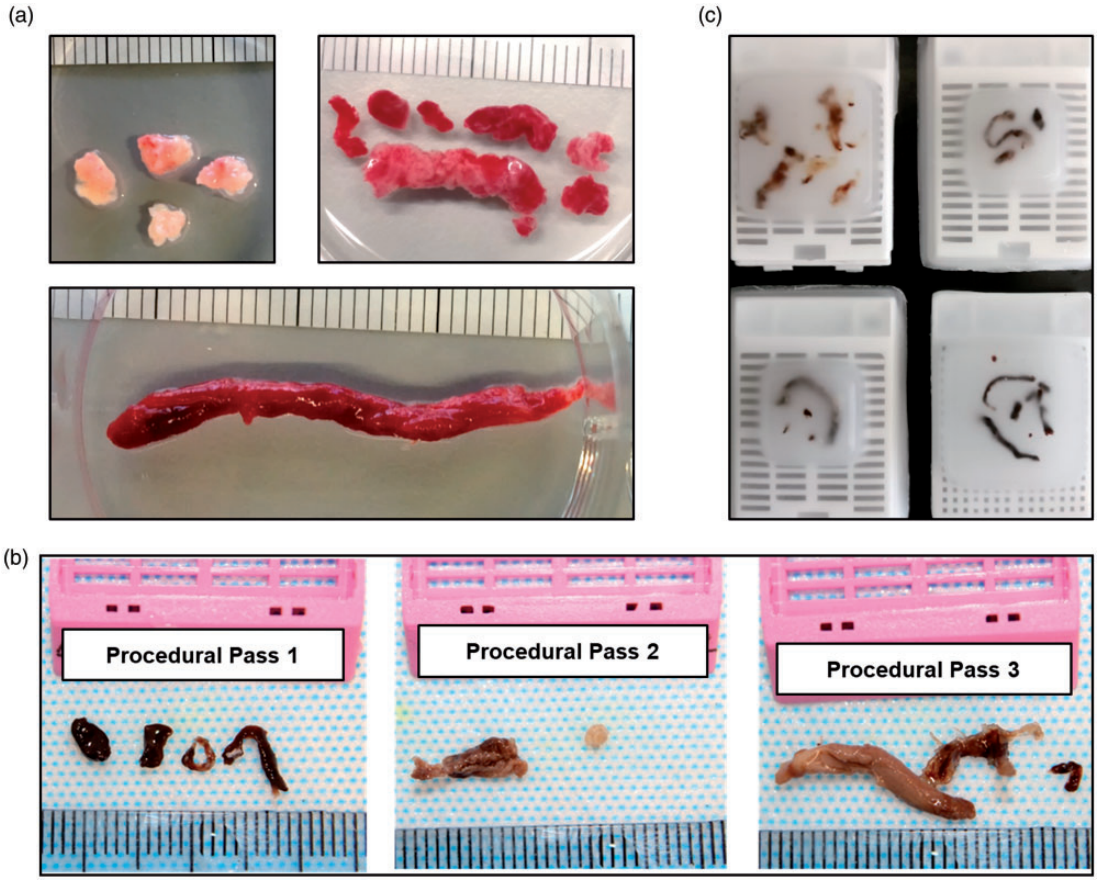
Processing step	Recommendation	Remarks
Retrieval	• Gently remove clot material from stent retriever and transfer to non-heparinized saline • Flush clot from aspiration device and transfer clot material to saline store in saline at 4°C for max. 24 h	
Fixation	• Fixate thrombus material in 10% formalin (3.7% formaldehyde) for 24 h	
Paraffin embedding	• Embed in paraffin via standard dehydration steps • Embed clot so that maximum cross section will be achieved when sectioning	
Sectioning	• Prepare sections of 3–5 μm thick	A total of 20 sections is typically sufficient to obtain a representative composition analysis
Staining	• Stain sections with ○ H&E (general structure) ○ MSB (fibrin, RBCs) ○ anti-CD42 antibody (platelets) ○ anti-CD45 antibody (WBC) ○ anti-VWF antibody (VWF)	MSB staining can be combined with a hematoxylin counterstain to visualize nucleated cells
Quantitative analysis	• Quantify the presence of clot components via computational color-based segmentation analysis • Analysis of one section per thrombus for each staining is sufficient in large scale studies. For small scale studies, three sections, from different parts throughout the thrombus can be recommended • Calculate for each component positive area as % of total thrombus section area	

predominantly on thrombi retrieved from stent retrievers, although aspiration retrievers are also used to remove thrombi (Supplemental File 1). Processing of the clot starts from the moment of clot retrieval. After retraction of the thrombectomy device, the retrieved clot material is gently removed from the device and transferred into saline (Figure 1(a)). It is recommended to use non-heparinized saline or saline free of other anticoagulants to prevent interference with the clot. For stent retrievers, the thrombus is entangled within the stent and needs to be carefully removed from the device using for example forceps. Depending on the fragility of the clot, it is either collected in one piece or in multiple fragments. For aspiration devices, clot material, together with aspirated blood, is flushed from the device into a collection container with saline. Since the aspirated blood can lead to further clot growth, isolated clots have to be immediately transferred into a different container with fresh saline until fixation.

Multiple procedural passes can give several fragments of clot material. Typically, all collected material from all passes in one patient are collected, processed, and labeled together as one and the same clot. If clot analysis per pass is desired, clot material from each pass can be processed separately (Figure 1(b)). Such approach can yield useful "per pass" information and thus insight into the composition of difficult to remove clots and clot fragments. It, however, significantly increases the workload involved.

Removal of the clot from the retriever allows for easy pooling of collected material and efficient further processing of the collected clot material. However, if the interaction between the clot and the stent struts is

Figure 1. (a) Thrombi retrieved after thrombectomy, prior to fixation, appear heterogeneous in size, color and fragmentation. Thrombi can also be analyzed in a per pass manner. (b) Thrombi retrieved can display marked heterogeneity per pass. (c) After fixation, thrombi are processed and embedded in paraffin blocks.



to be examined, the complete stent with the retrieved clot can be further processed as a whole.

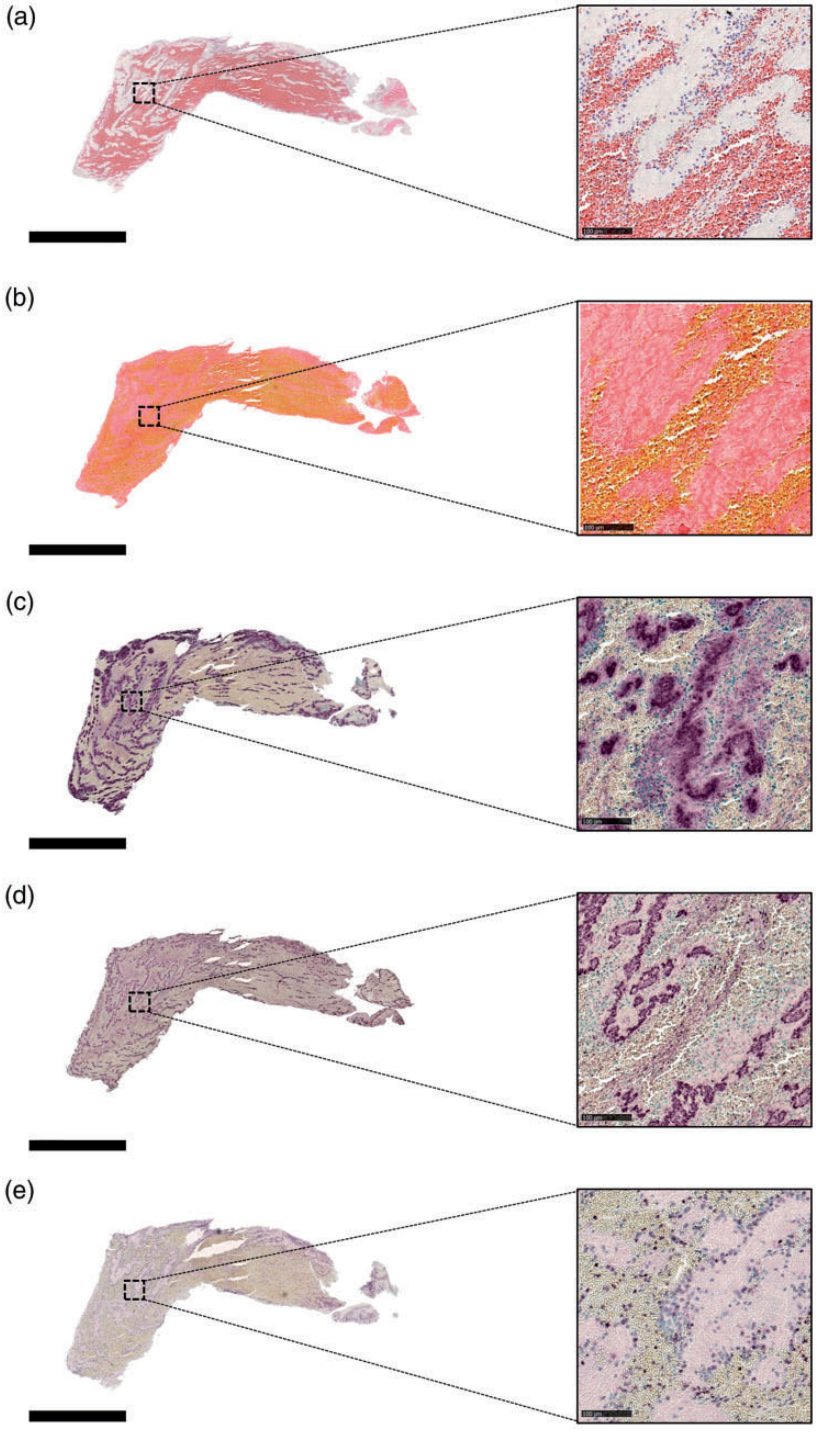
Fixation

After collection and rinsing of the clot in saline, the specimen needs to be fixated to preserve its structure. Although immediate fixation after thrombus retrieval and rinsing is recommended, our experience is that thrombi can be stored in saline up to 24 h without the occurrence of significant morphological changes in the thrombus. This time window allows protocol flexibility for thrombi that cannot be fixated immediately. For fixation, a solution of 10% formalin (3.7% formaldehyde) is routinely used (Supplemental File 1). If electron microscopy analyses are required, an appropriate fixation method for the different electron microscopy techniques is to be applied. We recommend fixation for 24 h. After fixation, it is recommended to start the embedding process. Longer fixation times are possible but can affect subsequent staining procedures by irreversibly masking antigens of interest. Our experience is that fixation protocols lasting longer than one week increase the autofluorescence of clot material, and we recommend that samples are stored in 10% formalin for no longer than one month, if they cannot be further processed after 24 h of fixation. If further processing of the clot is not feasible at the site of thrombectomy, the clot can be transported in a sealed container containing 10% formalin or saline for further processing. In addition, if mechanical analyses on the clot are to be performed, fixation should be avoided.

Paraffin embedding

After fixation, samples are embedded in paraffin. This results in good preservation of tissue morphology and allows easy long-term storage of the embedded clot material at room temperature until sectioning. We

Figure 2. Recommended histological staining procedures for thrombi retrieved from acute ischemic stroke thrombi. Consecutive thrombus sections were stained with (a) classical H&E, (b) MSB and antibodies against (c) CD42b (platelets), (d) VWF and (e) CD45 (leukocytes). Left panels (scale = 2 mm) represent an overview of the complete thrombus section. Right panels (scale = 100 μm) show a magnification. Classical H&E provides a general overview of the thrombus. On MSB staining, red areas show the presence of fibrin, whereas red blood cells are depicted in yellow. Positive signal in an immunohistochemical staining (c–e) is depicted in purple, red blood cells and nuclei are depicted in light brown and green, respectively.



recommend standard dehydration and paraffin embedding procedures, including sequential dehydration via increasing concentrations of ethanol (70%, 80%, 95%, 3 × 100%), followed by two immersions in xylene, and two immersions in paraffin. We recommend that the clot is embedded such that the largest cross-sectional area will be achieved, typically along the length of the thrombus (Figure 1(c)). All retrieved material from one patient can be embedded in one block, or material from each pass can be kept separate and embedded in separate blocks if per pass information is desired. Paraffin blocks containing stroke thrombi can be easily and safely transported to other research centers for further study.

Sectioning

In previous studies, section thickness has varied from 2 to 10 μm (Supplemental File 1) but a thickness of 4 to 5 μm is mainly used. Thinner sections can minimize the complication of several structures lying on top of each other and facilitate ease of quantification of main components by image analysis software, but require more skill to section. We recommend a thickness of 3 to 5 μm as optimum.

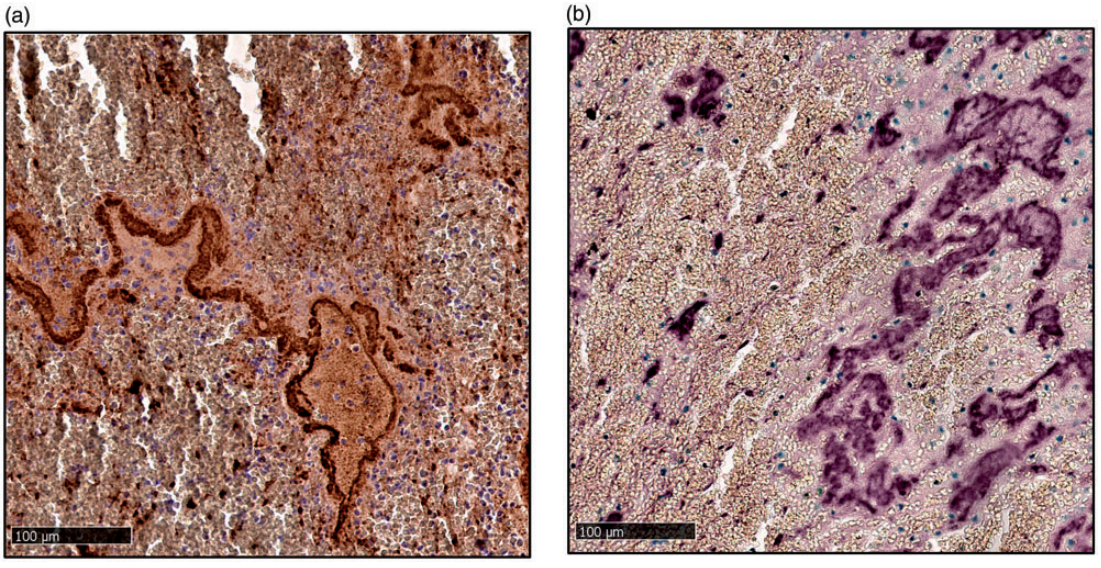
The paraffin block should be trimmed until a full-face section of the clot material is reached and serial sections taken from this point. Depending on the size of the clot fragment, multiple serial sections can be added to each slide. The total number of sections taken will depend on the number of tests and replicates being performed.

It is our experience that the composition of embedded thrombus does not vary considerably from superior to inferior aspect, suggesting that the whole thrombus may not need to be sectioned, depending on the research question. We suggest that, for standard analyses, the first 20 whole-face sections may be sufficient (see further information on intra-thrombus variability).

Staining

After sectioning, thrombus composition can be studied via various staining procedures. Depending on the component of interest, hematoxylin and eosin staining (H&E), Martius Scarlet Blue (MSB) staining, and immunohistochemistry procedures have been typically used. A detailed literature overview of histological procedures can be found in Supplemental File 1. Most studies have been focusing on the presence of red blood cells, fibrin, platelets, and white blood cells (Supplemental File 1). However, different staining methods with various degrees of specificity have been used per composition marker, which can impede easy comparison of the results. Quantification of thrombus composition is preferably based on staining procedures that have good specificity.

Figure 3. Comparison between various immunohistochemical substrate colors in stroke thrombi. (a) Development of a VWF staining using DAB (brown) with a hematoxylin counterstain. (b) Development of a VWF staining using a purple substrate with a methyl green counterstain. The purple substrate allows for better discrimination between positive staining and unstained red blood cells (light brown).



Classical H&E is mostly used to visualize the general overall structure of the thrombus. Staining with H&E stains acidophilic components (e.g. cytoplasm, fibrin) pink and basophilic components (e.g. nuclei) blue (Figure 2(a)). Although not very specific, we do recommend including the use of H&E as a quick and easy procedure for general purpose but not for quantitative determination of thrombus composition due to a lack

Figure 4. (a) Quantitative example of an MSB staining using color-based segmentation analysis in Image J. In panel a, an image of the staining before application of a threshold is shown (left), as well as the same image after applying a threshold for fibrin (middle) and for red blood cells (right). (b) Quantitative example of an MSB staining using color-based machine learning techniques in Orbit Image Analyzer before (left) and after (right) software analysis. (c) Quantitative example of a VWF staining using color-based segmentation analysis in Image J before (left) and after (right) applying and selecting the color-based segmentation.

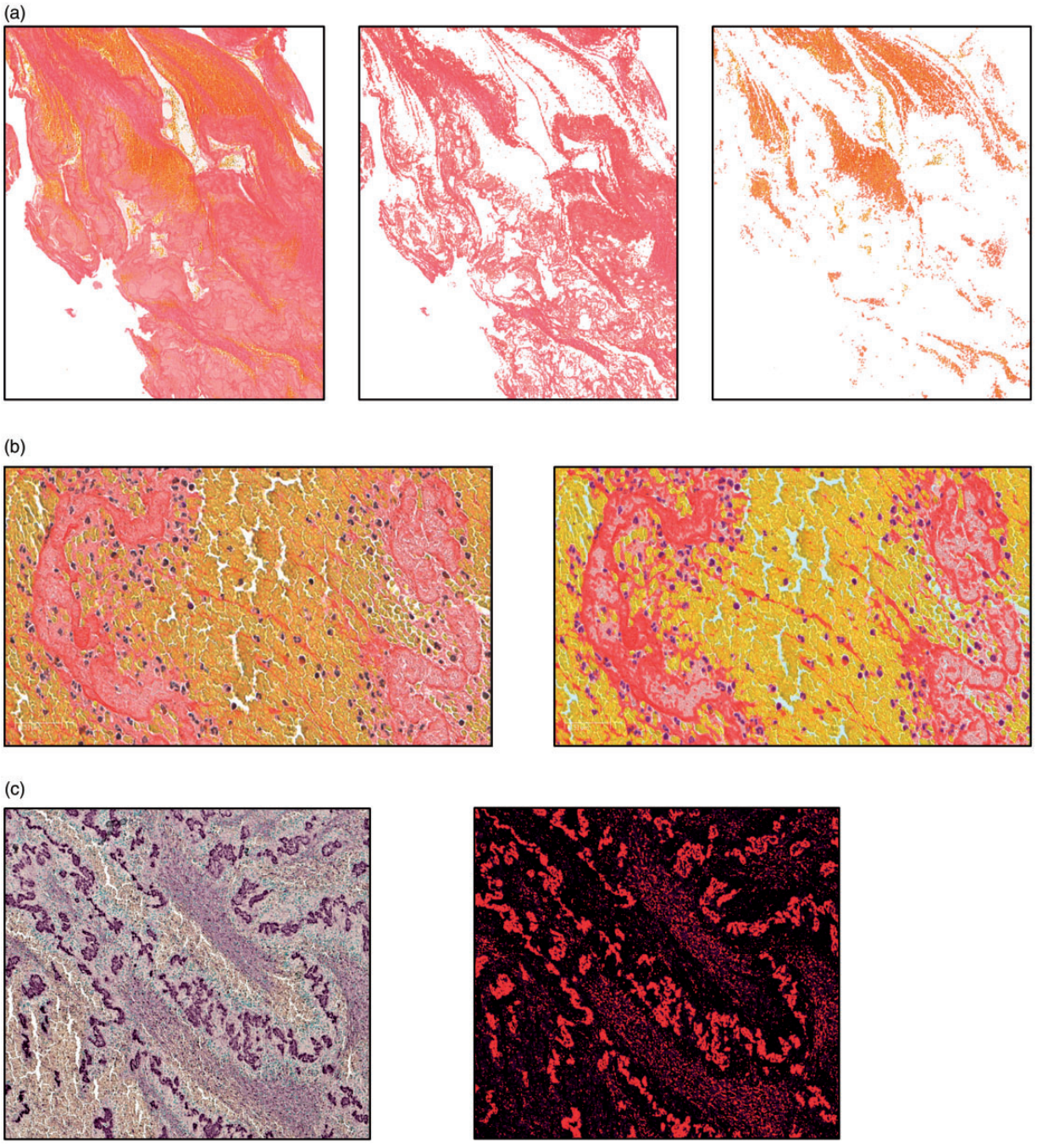
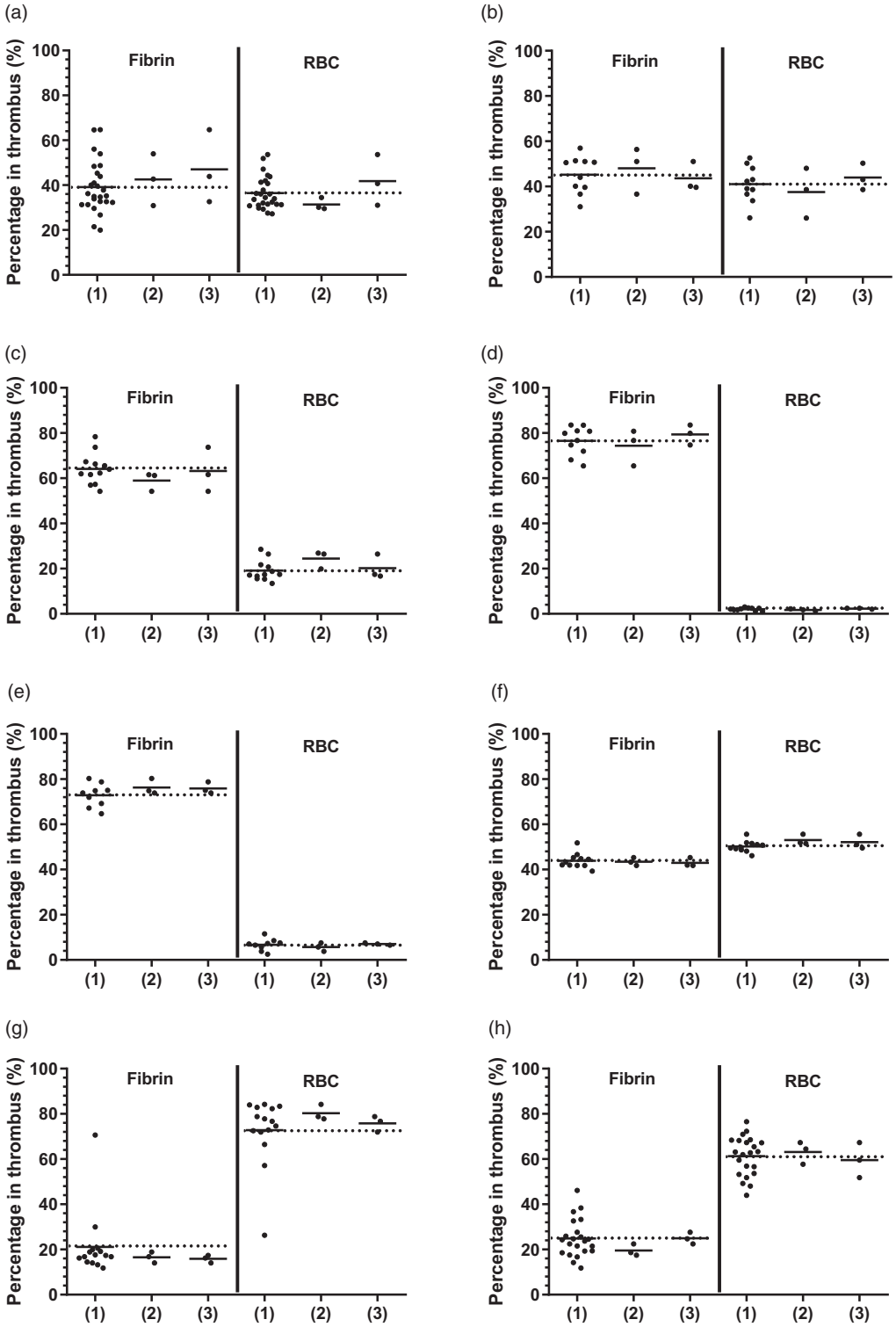


Figure 5. (a–h) Intra-thrombus variability measured in eight stroke thrombi stained with MSB and analyzed for the amount of fibrin and red blood cells. Percentages of fibrin and red blood cells in the thrombus are shown for all sections analyzed throughout the entire thrombus (1) (approximately every 75 μm, each data point represents one section), or when only three sections, located at the end of the first, second and third quarter of the thrombus, were analyzed, representing a scenario in which not all sections are stained (2) or when the first three sections that are 75 μm apart were analyzed, representing a scenario in which only the first part of the thrombus is sectioned (3).



of specificity. Instead, we recommend Martius Scarlet Blue (MSB) staining for quantification of RBC and fibrin content (Figure 2(b)). MSB staining allows for the selective demonstration of fibrin, which is stained red using a crystal scarlet solution and of RBC that are stained yellow using a Martius yellow solution. Although not common in stroke thrombi, collagen appears blue with a methyl blue solution. The distinct yellow and red colors are easy to discriminate and allow for a quick, cost-effective and selective determination of RBC and fibrin, which is practical for large-scale, high-throughput studies.

Besides RBC and fibrin, we believe that three other components are of general interest and should become standard for thrombus analysis. These are white blood cells (WBC), platelets, and von Willebrand factor (VWF), which each are best visualized through immunohistochemical staining procedures (Figure 2(c) to (e)).

For platelets and white blood cells, antibodies against various epitopes are available but the consensus is that CD42 and CD45 are the most specific epitopes for platelets and white blood cells, respectively. Commercial antibodies against VWF are available and give reliable results on VWF content.[9] Additional antibodies, e.g. against different subsets of leukocytes or other proteins of interest, can be added. Immunohistochemical stains are typically based on the precipitation of 3,3'-diaminobenzidine (DAB), a substrate with a brown color in combination with a hematoxylin counterstain. In our hands, this method often gives unsatisfactory results because the specific brown staining of the antigen is not easily discriminated from unstained red blood cells that also have a brownish color (Figure 3(a)). In order to perform accurate color-based segmentation quantification, we recommend the use of other colored substrates, such as a purple-colored substrate in combination with a methyl green counterstain (Figure 3(b)).

Analysis

Besides uniform staining procedures, standardized quantitation algorithms to calculate thrombus composition are also important to improve generalizability in the field. In the past, both manual quantifications and color-based segmentation analysis have been used (Supplemental File 1). Manual quantification is primarily used to count the number of cells per mm^2, but can be labor-intensive, especially in large-scale studies. Computational color-based segmentation analysis provides a better way to obtain quantitative information on thrombus composition (RBC, fibrin, WBC, platelets, and VWF). Various software programs such as ImageJ, Adobe Photoshop and Orbit Image Analyzer are available to apply thresholds for color and good selective quantification of specific colors/components (Figure 4(a) to (c)).[10–13] These software-based analyses provide data in a partially automated manner and produce quantitative results (% of total thrombus section area) that are easy to compare across multiple studies/centers. In our experience, software-based computational analysis produces thrombus composition data that strongly correlate with manual quantification methods. The very considerable time saving makes software-based analysis a very attractive option.

A key question performing quantitative analysis is whether intra-thrombus heterogeneity, and thus the location of the section within the thrombus, affects quantitative conclusions. Macroscopically, thrombi appear heterogeneous in size, color, shape, and fragmentation. Most quantitative studies have taken the data obtained from only one section per thrombus as representative for the whole thrombus. To better understand intra-thrombus variability, we completely sectioned eight thrombi from ischemic stroke patients and analyzed multiple sections throughout the thrombus (approximately 75 μm apart). We used MSB staining to quantify the presence of fibrin and RBC on each section (Figure 4). As expected, in each of the eight thrombi, some degree of intra-thrombus variation is observed throughout the thrombus but measurements from different sections of one thrombus cluster around the mean value with an acceptable spread. Sectioning the whole thrombus to analyze multiple sections throughout the clot might give a more accurate of the mean value of the composition but is not practical for larger studies. Partial sectioning of the thrombus, combined with analysis of only one to three sections reduces the workload and still provides a good estimate of thrombus composition, as shown in Figure 5. A similar approach was used to assess the intra-thrombus variability of the presence of VWF and platelets (via immunostaining) in three thrombi, yielding similar results (data not shown). Based on our experience, we recommend the use of one thrombus section per staining to estimate thrombus composition in large-scale studies, as large sample sizes will compensate the minor intra-thrombus variability. For small sample size studies and testing of novel markers, we do recommend the use of three sections per staining to account for potential intra-thrombus variability (Figure 5(a) to (h)).

Collection of patient data

Understanding thrombus composition has a number of potential implications for clinical treatment. Despite recent advances in endovascular procedures, still 20% of thrombi are not retrievable.[14] Although these thrombi are not available for analysis, linking thrombus composition to both clinical and procedural

information can provide useful insights that can guide stroke therapy. For example, fibrin-rich thrombi have been associated with an increased coefficient of friction, a higher number of retraction maneuvers, and increased thrombus extraction times.[15–17] Evidently, linking thrombus composition with other clinical aspects such as stroke etiology and stroke outcome can provide crucial information. Therefore, when collecting thrombi for histological analysis, we recommend keeping a general data abstraction form that includes both clinical and procedural information per thrombus. To prevent a selection bias, we also recommend collecting the clinical information of those thrombi that are not retrievable (TICI 0 or where no thrombus material was found in the stent). Supplemental file 2 provides an example of a data abstraction form that contains the 'must-have' data. So far, thrombus composition studies have reported a wide variety of clinical and procedural information with a particular focus on stroke severity (National Institute of Health Stroke Scale; NIHSS), recanalization success (Thrombolysis in Cerebral Infarction Scale; TICI), stroke etiology (Trial of Org 10172 in Acute Stroke Treatment; TOAST), functional outcome (modified Ranking Score; mRS), and procedural information (amount of retraction maneuvers and thrombus extraction time). In addition, we recommend the collection of complete blood counts as well as other clinical parameters such as for example comorbidities in order to have a detailed clinical history of the patient (Supplemental File 2). Standardization of the 'must-have' information (Supplemental file 2) will increase generalizability among studies. Record keeping of this information must be embedded in the clinical standard operating procedures of the thrombectomy unit. The move towards the development of national stroke registries in Europe will also facilitate correlation between thrombus composition and clinical information.

Limitations

The analysis of thrombi retrieved from ischemic stroke patients has some limitations that are worth consideration. Rolling, movement, or fragmentation of the thrombus in the thrombectomy device during the extraction procedure hinder exact knowledge on the original orientation of the thrombus in the occluded blood vessel. Importantly, only those thrombi that did not dissolve spontaneously or after administration of tissue plasminogen activator (t-PA) and thrombi that could be successfully retrieved via thrombectomy are accessible for study. This impedes the assessment of thrombi that were t-PA susceptible or thrombectomy resistant. Nevertheless, specific attention to those thrombi that were difficulty to retrieve could provide useful information on thrombi that are impossible to retrieve.

Conclusion

In this rapidly evolving field, best practices for thrombus characterization and data collection will become important. This effort will maximize the generalizability of the results to make important in roads with respect to discrimination of stroke etiology and informed secondary prevention measures, as well as optimizing the thrombectomy procedure to maximize clinical outcomes.

Declaration of conflicting interests

The author(s) declared the following potential conflict of interest with respect to the research, authorship, and/or publication of this article: F.C. declares conflict of interest with Medtronic, Guerbet, Balt Extrusion (speaker honorarium), Codman Neurovascular (core lab). W.H. declares honoraria from Cerenovus for participation in advisory boards and steering committees. T.A. declares consultancy for Ablynx, Amnis Therapeutics, Anaconda, Cerenovus, Medtronic and Rapid Medical. W.B. has received research grants from the National Institute of Health, has an Owership Interest in Marblehead Medical LLC and has received research support from Johnson & Johnson. A.C.G.M.v.E, D.S.L., F.D., I.S., K.M.D., M.J.G., S.F., S.F.D.M, S.S. have no conflict of interest to declare.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study under the approval of the AZ Groeninge Hospital ethical committee (AZGS2015065). No animal experiments were performed in this study.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship and/or publication of this article: This work was supported by research grants to S.F.D.M. from the Fonds voor Wetenschappelijk Onderzoek – Vlaanderen (FWO) (research grants G.0A86.13, G.0785.17 and 1509216N), the KU Leuven (OT/14/099 and ISP/14/02L2), the Queen Elisabeth Medical Foundation and by the European Union's Horizon 2020 Research and Innovation Program INSIST under grant agreement No 777072. F.D. is a postdoctoral fellow of the FWO (FWO, 12U7818N). This work is also supported by a research grant to KD from Science Foundation Ireland, co-funded under the European Regional Development fund under Grant Number 13/RC/2073 and Cerenovus.

ORCID iD

S Staessens https://orcid.org/0000-0001-5530-6961

Supplemental material

Supplemental material for this article is available online.

References

1. Benjamin EJ, Blaha MJ, Chiuve SE, et al. Heart disease and stroke statistics – 2017 update: a report from the American Heart Association. *Circulation* 2017; 135: e146–e603.
2. Berkhemer OA, Fransen PSS, Beumer D, et al. A randomized trial of intraarterial treatment for acute ischemic stroke. *N Engl J Med* 2015; 372: 11–20.
3. Campbell BCV, Mitchell PJ, Kleinig TJ, et al. Endovascular therapy for ischemic stroke with perfusion-imaging selection. *N Engl J Med* 2015; 372: 1009–1018.
4. Goyal M, Demchuk AM, Menon BK, et al. Randomized assessment of rapid endovascular treatment of ischemic stroke. *N Engl J Med* 2015; 372: 1019–1030.
5. Saver JL, Goyal M, Bonafé A, et al. Stent-retriever thrombectomy after intravenous t-PA vs. t-PA alone in stroke. *N Engl J Med* 2015; 372: 2285–2295.
6. Jovin TG, Chamorro A, Cobo E, et al. Thrombectomy within 8 hours after symptom onset in ischemic stroke. *N Engl J Med* 2015; 372: 2296–2306.
7. De Meyer SF, Andersson T, Baxter B, et al. Analyses of thrombi in acute ischemic stroke: a consensus statement on current knowledge and future directions. *Int J Stroke* 2017; 12: 606–614.
8. Brinjikji W, Duffy S, Burrows A, et al. Correlation of imaging and histopathology of thrombi in acute ischemic stroke with etiology and outcome: a systematic review. *J Neurointerv Surg* 2017; 9: 529–534.
9. Denorme F, Langhauser F, Desender L, et al. ADAMTS13-mediated thrombolysis of t-PA-resistant occlusions in ischemic stroke in mice. *Blood* 2016; 127: 2337–2345.
10. Prasad K and Prabhu GK. Image analysis tools for evaluation of microscopic views of immunohistochemically stained specimen in medical research – a review. *J Med Syst* 2012; 36: 2621–2631.
11. Lehr HA, van der Loos CM, Teeling P and Gown AM. Complete chromogen separation and analysis in double immunohistochemical stains using Photoshop-based image analysis. *J Histochem Cytochem* 1999; 47: 119–126.
12. Zhou Y, Ru GQ, Yan R, et al. An inexpensive digital image analysis technique for liver fibrosis quantification in chronic hepatitis B patients. *Ann Hepatol* 2017; 16: 881–887.
13. Arena ET, Rueden CT, Hiner MC, et al. Quantitating the cell: turning images into numbers with ImageJ. *Wiley Interdiscip Rev Dev Biol* 2017; 6: e260.
14. Yoo AJ and Andersson T. Thrombectomy in acute ischemic stroke: challenges to procedural success. *J Stroke* 2017; 19: 121–130.
15. Maekawa K, Shibata M, Nakajima H, et al. Erythrocyte-rich thrombus is associated with reduced number of maneuvers and procedure time in patients with acute ischemic stroke undergoing mechanical thrombectomy. *Cerebrovasc Dis Extra* 2017; 8: 39–49.
16. Boeckh-Behrens T, Schubert M, Förschler A, et al. The impact of histological clot composition in embolic stroke. *Clin Neuroradiol* 2016; 26: 189–197.
17. Gunning GM, Kevin M, Mirza M, et al. Clot friction variation with fibrin content; implications for resistance to thrombectomy. *J Neurointerv Surg* 2018; 10: 34–38.