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Novel human acute ischemic stroke blood clot analogues for in-vitro thrombectomy testing

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Video S1 - clot ingestion.mp4
Video S2 - clot pull by suction catheter with embolization.mp4
Video S3 - clot pull by stent retriever with embolization.mp4
Novel human acute ischemic stroke blood clot analogues for *in-vitro* thrombectomy testing

Seán T. Fitzgerald¹,², Yang Liu¹, Daying Dai¹, Oana M. Mereuta¹,²,³, Mehdi Abbasi³, Jorge L. Arturo Larco⁴, Andrew S. Douglas²,³, David F. Kallmes¹, Luis Savastano⁴, Karen M. Doyle²,³, Waleed Brinjikji¹,⁴

¹Department of Radiology, Mayo Clinic, Rochester, MN, USA, ²Department of Physiology, National University of Ireland Galway, Galway, Ireland, ³CÚRAM – SFI Centre for Research in Medical Devices, National University of Ireland Galway, Galway, Ireland, ⁴Department of Neurosurgery, Mayo Clinic, Rochester, MN, USA

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**Corresponding Author:**
Dr Seán Fitzgerald

**Address:** Department of Physiology, National University of Ireland Galway, Galway, Ireland,

**Email:** Sean.Fitzgerald@nuigalway.ie

**Telephone:** +353-91-495397

**Fax:** 507-255-0706

**Twitter:** @FitzSeanT
Abstract:

Background and Purpose: Previous studies have successfully created blood clot analogues for *In-vitro* endovascular device testing using animal blood of various species. Blood components vary greatly among species, therefore, creating clot analogues from human blood are likely a more accurate representation of thrombi formed in human vasculature.

Materials and Methods: Following approval from Mayo Clinic Institutional Review Board, human whole blood and platelet donations were obtained from the Blood Transfusion service. 12 clot analogues were created by combining different ratios of RBC+Buffy coat, Plasma, and Platelets. Thrombin and CaCl₂ were added to stimulate coagulation. Clot composition was assessed using histological and Immunohistochemical staining. To assess the similarities of mechanical properties to patient clots, three types of clot analogues (soft, elastic, and stiff) were selected for in-vitro thrombectomy testing.

Results: The range of histopathological compositions produced is representative of clots removed during thrombectomy procedures. The RBC composition ranged from 8.9%-91.4% and Fibrin composition ranged from 3.1%-53.4%. Platelet (CD42b) and von Willebrand Factor (vWF) ranged from 0.5%-47.1% and 1.0%-63.4%, respectively. The soft-clots had the highest first-pass-effect and successful revascularization rates followed by the elastic and stiff clots. Distal embolization events were observed when clot ingestion cannot be achieved, requiring device-pullback. Incidence rate of distal embolization is the highest for the stiff clots due to the weak clot/device integration.

Conclusions: RBC-rich, Fibrin-rich, and Platelet-rich clot analogues that mimic clots retrieved from Acute Ischemic Stroke Patients were created *In-vitro*. Differing retrieval outcomes were confirmed using in-vitro thrombectomy testing in a subset of clots.
**Abbreviations**

DA: Direct Aspiration

DE: Distal Embolization

FFPE: Formalin-fixed paraffin-embedded

FPE: First Pass Effect

MSB: Martius Scarlett Blue

RBCs: Red Blood Cells

STR: Stentriever

SR: Successful Revascularization

WBCs: White Blood Cells
Introduction:

In the treatment of acute ischemic stroke, the achievement of complete revascularization from a single mechanical thrombectomy attempt, termed First Pass Effect (FPE), is associated with significantly improved outcomes for patients\(^1\(^\)–\(^2\). Removing the clot in a fragmented manner increases the potential of embolization to new territories, a major contributing factor to poor neurological outcomes due to additional brain infarction\(^3\)--\(^5\). Despite the advancement in the second generation mechanical thrombectomy devices, the rates of FPE remain low, as low as 29% in the recently reported Aspiration versus Stent Retriever (ASTER) trial\(^6\).

Previous studies have demonstrated that a wide variety of occlusive clots can cause an LVO\(^7\)--\(^11\) and clot composition has been shown to have a significant impact on the success of mechanical thrombectomy procedures\(^7\),\(^12\),\(^13\). This suggests that in order to further advance the success rates of stroke intervention, we must turn our attention to clot composition and compare treatment strategies using in-vitro thrombectomy models of the cerebral vasculature. Previous studies have successfully created blood clot analogues for In-vitro testing using animal blood of various species which have significantly advanced our understanding of clot biomechanics and imaging characteristics\(^13\)--\(^19\). However, blood components and blood groups vary amongst species\(^20\) and thus, creating clot analogues with human blood is likely a more accurate representation of thrombi formed in the human vasculature.

The hypothesis of the study is that the diverse range of clots retrieved from AIS patients can be accurately replicated using human blood by mimicking the process by which clots form In-vivo. The rationale for this study is that, as the success of mechanical thrombectomy procedures is influenced by the composition of the clot, creating human clot
analogs that accurately represent the different phenotypes retrieved from patients and testing them in an in-vitro thrombectomy system will allow us to compare the performance of different thrombectomy devices and techniques. We will thereby be able to determine the optimum treatment approach for each clot phenotype, thereby optimizing the chances of achieving the desired First Pass TICI3 outcome in the clinical setting\(^1\). To assess the similarities of mechanical properties to patient clots, three types of clot analogues (soft, elastic, and stiff) were selected for in-vitro thrombectomy testing.

**Materials and Methods:**

**Human Clot Analogue Creation**

This Study received Institutional Review Board approval from Mayo Clinic Rochester in accordance with the ethical standards of the Declaration of Helsinki. A total of 12 clot analogues types were created as per Table 1. These clots analogues were selected to be representative of the previously identified phenotypes of clots retrieved from AIS patients; including; RBC-Rich, Fibrin-Rich, and Platelet-Rich clots\(^2\).

A human whole blood donation and a human platelet donation from two separate donors were obtained from the Mayo Clinic Blood Transfusion Service. The whole blood was centrifuged at 1,200RPM for 20 minutes at 20°C to separate it into its constituents\(^2\). Plasma was harvested by pipetting and the remaining Red Blood Cells and Buffy Coat were mixed together by inverting. Plasma and Platelets were combined first as per the table and then then 3 \(\mu\)l of Thrombin (1NIH/mL, Roche Diagnostic GmbH, Mannheim, Germany, #T6884) was added to activate platelets for a total of 1-2 minutes whilst continuously mixing. 300 \(\mu\)l of 5\% CaCl\(_2\) (Sigma Aldrich, St. Louis, Missouri, United States, #C1016) solution is then added followed by the RBC+Buffy Coat mixture. The tube was then quickly mixed by inversion 5
times and then the clot analogue mixture was drawn into a 3mL syringe. The syringes were spun overnight at 20RPM at room temperature to mimic the dynamic flow conditions of the human vasculature.

**Table 1: Volume of components added to each clot analogue type**

| Ratio    | Platelets (µl) | Plasma (µl) | RBC+Buffy Coat (µl) |
|----------|----------------|-------------|---------------------|
| **Plasma Only** |                |             |                     |
| 1:5      | 0µL           | 2,400µL     | 600µL               |
| 1:10     | 0µL           | 2,700µL     | 300µL               |
| 1:50     | 0µL           | 2,940µL     | 60µL                |
| 1:100    | 0µL           | 2,970µL     | 30µL                |
| **Platelets Only** |            |             |                     |
| 1:5      | 2,400µL       | 0µL         | 600µL               |
| 1:10     | 2,700µL       | 0µL         | 300µL               |
| 1:50     | 2,940µL       | 0µL         | 60µL                |
| 1:100    | 2,970µL       | 0µL         | 30µL                |
| **Plasma and Platelets** |            |             |                     |
| 1:5      | 1,200µL       | 1,200µL     | 600µL               |
| 1:10     | 1,350µL       | 1,350µL     | 300µL               |
| 1:50     | 1,470µL       | 1,470µL     | 60µL                |
| 1:100    | 1,485µL       | 1,485µL     | 30µL                |

*3µL of Thrombin (1NIH/mL) and 300µL of 5% CaCl\(_2\) were added to stimulate coagulation.

**Patient Cohort**

Clots were collected from 100 patients who underwent mechanical thrombectomy for the treatment of acute ischemic stroke at Mayo Clinic Rochester. Where more than one procedural pass was needed to retrieve the occlusive clot, all fragments of clot were combined for histological analysis. The inclusion criteria were; >18 years, having undergone mechanical thrombectomy treatment for acute ischemic stroke and with clot material available for analysis. A waiver of informed consent was granted for the purposed of collecting retrieved clot material from acute ischemic stroke patients for this study.
Histological Processing and Staining

Gross photos were taken of each clot and analogue before fixation overnight in 10% phosphate-buffered formalin. All clots and analogues were then processed using a standard tissue processing protocol and embedded in paraffin. The formalin-fixed paraffin-embedded (FFPE) material was cut into 3µm sections. The Martius Scarlett Blue stain is now regarded as the gold-standard for assessing clot composition as it identifies platelet-rich regions of thrombi in addition to RBCs, WBCs and Fibrin\textsuperscript{21, 23}. Two representative slides were stained with Martius Scarlett Blue (MSB) to identify the common clot constituents; RBCs, White Blood Cells, Fibrin, Platelets/other, Collagen and Calcification as described previously\textsuperscript{21, 23}. Clot phenotype for both the clinical samples and the clot analogs was defined based on the dominant component (%) in each clot as determined by the MSB histological staining.

Immunohistochemistry

Platelets and vWF levels are useful additional hallmarks of clot composition\textsuperscript{24-26}. Immunohistochemical staining for platelets (CD42b) and von Willebrand Factor (vWF) was performed on a Leica Bond RX autostainer. Antigen retrieval with Tris-EDTA was performed for platelet staining (anti-CD42b); no antigen retrieval was used for vWF staining. Primary antibody (anti-CD42b; Abcam ab27669, 1:200 dilution, anti-vWF; Dako A-0082, 1:200 dilution); incubation time was 30mins. Negative controls were performed by omission of the primary antibody step. A Leica BOND Polymer Refine Red Detection system that incorporates a post primary antibody, polymer reagent and Fast Red chromogen, and hematoxylin counterstain was used for visualization (Leica Biosystems). Sections were washed in warm soapy water, dehydrated in increasing alcohol gradients, cleared in xylene and mounted with DPX.
**Imaging and Quantification**

Following staining, a representative slide of each stain was scanned at 20x magnification (Motic Easyscan Pro, Motic Digital Pathology). Histologic quantification was performed on the digital slide using Orbit Image Analysis Software (www.Orbit.bio) as described previously. Percentage area of each component (RBC, WBC, Fibrin and Platelet/other) within the clot was calculated for the histological staining with MSB. Percentage area of positive IHC staining was calculated separately for CD42b and vWF.

**Thrombectomy Testing in a Benchtop Stroke Platform**

The mechanical properties of clots vary based on their histological composition; clot analogues with an increasing volume of Platelets contract to a greater degree due to the force of platelet contraction, resulting in stiffer clot analogues. Clots analogs that have a high RBC content will typically be softer, more friable clots and clots made from plasma only, will produce clot analogs with a network of thin Fibrin-stands. Three phenotypes of clot analogues with varying compositions of RBCs, Plasma and Platelets were selected to represent prominent phenotypes of clots retrieved from AIS patients during thrombectomy; Soft (1:10 RBC + buffy coat: Plasma Only), Elastic (1:5 RBC + buffy coat:platelets + plasma), and Stiff (1:10 RBC + buffy coat:platelets + plasma).

Thrombectomy testing were carried out on these clots inside a benchtop stroke platform as previously described. Briefly, a cerebrovascular glass model, where the lumen resembles the intracranial internal carotid artery, the anterior cerebral artery, and the middle cerebral arteries, is connected to a customized flow system to deliver flow with physiologically representative flow rate and pressure. Clot analogues measuring 6mm in length were introduced into the flow system and embolized to the M1-M2 bifurcation.
Revascularization was carried out using: 1) the direct aspiration (DA) technique with the Sofia 6F aspiration catheter, and 2) stent retriever with aspiration (SR+A) technique with the Solitaire stent retriever and the Sofia 6F aspiration catheter. For each type of clot analogue and revascularization technique, five clot analogs were made to replicate large vessel occlusion (LVO) stroke. For each LVO case, 3 device passes were attempted before declaring failure. Revascularization result, number of passes, and embolization events were recorded for each test.

Ingestion was defined as complete ingestion of the clot into the catheter, First Pass Effect was defined as complete removal of the clot from the target artery in the first procedural pass, Successful Recanalization (SR) was defined as the as complete removal of the clot from the target artery within 3 procedural passes and Distal Embolization was defined as the occurrence of visible fragments of clots being dislodged and migrating distally from the target vessel. The thrombectomy processes were recorded and the failure mechanism, including the presence of Distal Embolization, were confirmed following the procedure.

**Statistical analysis**

All statistical correlations were assessed and graphs were generated using GraphPad Prism 8. MSB histological composition was reported as % of the total clot area, positive immunohistochemistry staining (CD42b & vWF) was reported as % of the total clot area. A Shapiro-Wilk test indicated that quantitative variables did not follow a standard normal distribution. The non-parametric Spearman rho correlation was used to assess the similarity between clot analogues and clinical samples.
**Results:**

*Clot Analogue Appearance*

The gross appearance of each of the human clot analogues after clot formation and also post fixation in 10% Neutral buffered formalin is show in Figure 1. Clots analogues that are rich in Red Blood cells clots (e.g. 1:10 RBC+Buffy Coat:Plasma Only) have a dark red color after creation and a black color post-fixation. Clots that contain high platelet content (e.g. 1:100 RBC+Buffy Coat:Platelets Only) have white platelet-rich regions that are visible both pre and post fixation. Clots that are Fibrin-rich but not platelet-rich have a light red color after creation and a brownish color post-fixation (e.g. 1:50 RBC+Buffy Coat:Plasma Only). The Platelet-rich clots are smaller in clot volume due to the effect of platelet stimulated contraction of the clots. The clot analogues produced have a similar gross appearance to clots extracted from patients during mechanical thrombectomy procedures for the treatment of AIS.
Figure 1: Gross Photographs of Human Blood Clot Analogues Pre and Post Fixation. Gross Photographs of Human Blood Clot Analogues of various compositions were taken pre and post fixation in PFA. Clot analogues were created using different ratios (1:5, 1:10, 1:50 and 1:100) of RBC+Buffy Coat and Plasma Only, Platelets Only and Plasma+Platelets.
**Histological Composition**

The MSB stain was used to assess the histological composition of the clot analogues (Table 2) and of the clots retrieved from AIS patients (Figure 2). Red Blood cell-rich, Fibrin-rich, and Platelet-rich clot analogues that mimic clots retrieved from acute ischemic stroke patients were created. The range of histopathological compositions of the clot analogues is similar to that of the clinical samples (Figure 2). The addition of a large volume of Red Blood Cells leads to a RBC-Rich clot regardless of whether platelets and/or plasma were also added (Figure 2). The Red blood cell composition of the clot analogs ranged from 8.9% to 91.4% and the clots retrieved from the patients ranged from <1% to 85%, there was a significant positive correlation between the RBC composition of the analogs and the clinical samples ($r_s=.755$, $p=0.010^*$). The platelet composition of the clot analogs ranged from 5.4% to 83.7% whilst the clinical samples ranged from 3% to 88% ($r_s=618$, $p=0.048^*$). Fibrin composition of the clot analogs ranged from 3% to 53% and from 3% to 77% in the clinical samples ($r_s=136$, $p=0.694$). White blood cell are typically a minor component of clots and account for an average of 3.5% of clinical clots and 1% of clot analogs ($r_s=.311$, $p=0.345$). Each of these components is in line with the composition of acute ischemic stroke clots, reported previously in the literature$^{21, 30, 31}$.
Figure 2: Histological Quantification of MSB stained human clots retrieved from patients and human clot analogues created In-vitro. (A) The histological composition of various 100 clots retrieved from patients was assessed using the MSB stain and grouped according to their dominant component resulting in three main groups; Red Blood Cell-Rich, Fibrin-Rich and Platelet-Rich. (B) The histological composition of various human clot analogues created In-vitro was also assessed using the MSB stain and grouped according to their dominant component resulting in the same three phenotypes; Red Blood Cell-Rich, Fibrin-Rich and Platelet-Rich.
Figure 3: Histology and Immunohistochemical stained images of clots retrieved from an acute ischemic stroke patient and of a human clot analogue. A comparison of the similarity in terms of gross photographs, MSB stain, CD42b (Platelets) and von Willebrand Factor between (A) a clot retrieved from an acute ischemic stroke patient and (B) A human clot analogue (1:50 RBC+Buffy Coat : Platelets) is shown. MSB stain: Red Blood Cells (Yellow), White Blood Cells (Purple), Fibrin (Red), Platelets/Other (Grey), and Collagen (Blue). Platelets (CD42b) and Von Willebrand Factor; Positive staining is Red.
| Composition Ratio's | MSB Histological Stain | Immunohistochemistry |
|---------------------|------------------------|----------------------|
|                     | Red Blood Cells | White Blood Cells | Fibrin | Platelets and Other | CD42b | Tissue | vWF | Tissue |
| RBC+BuffyCoat : Plasma Only |            |                |       |               |       |       |     |        |
| 1:5                 | 78.84%      | 0.33%           | 7.61% | 13.22%        | 2.64% | 97.36%| 1.81%| 98.19%|
| 1:10                | 78.02%      | 0.07%           | 13.16%| 8.74%         | 1.44% | 98.56%| 1.05%| 98.95%|
| 1:50                | 28.67%      | 0.15%           | 42.55%| 28.63%        | 0.46% | 99.54%| 3.27%| 96.73%|
| 1:100               | 19.61%      | 0.04%           | 45.73%| 34.62%        | 3.03% | 96.97%| 1.18%| 98.82%|
| RBC+BuffyCoat : Platelets Only |            |                |       |               |       |       |     |        |
| 1:5                 | 59.22%      | 1.05%           | 12.59%| 27.15%        | 30.44%| 69.56%| 19.26%| 80.74%|
| 1:10                | 30.09%      | 0.91%           | 4.17% | 64.83%        | 47.11%| 52.89%| 63.43%| 36.57%|
| 1:50                | 15.33%      | 0.03%           | 5.87% | 78.77%        | 19.30%| 80.70%| 17.44%| 82.56%|
| 1:100               | 12.01%      | 0.03%           | 4.29% | 83.66%        | 25.35%| 74.65%| 34.65%| 65.35%|
| RBC+BuffyCoat : Platelets + Plasma |        |                |       |               |       |       |     |        |
| 1:5                 | 91.42%      | 0.06%           | 3.11% | 5.41%         | 22.73%| 77.27%| 3.49%| 96.51%|
| 1:10                | 77.13%      | 0.19%           | 13.17%| 9.51%         | 12.31%| 87.69%| 15.47%| 84.53%|
| 1:50                | 50.51%      | 0.01%           | 19.45%| 30.03%        | 12.01%| 87.99%| 10.81%| 89.19%|
| 1:100               | 8.90%       | 0.06%           | 53.39%| 37.64%        | 11.72%| 88.28%| 2.25%| 97.75%|
Immunohistochemical Composition

The composition of platelets (CD42b) varied from 0.5% to 47.1% of the total area and the composition of vWF varied from 1.1% to 63.4% of the total area (Table 1). Clot analogues made with platelets only had the largest proportion of both CD42b and vWF present while clot analogues made with plasma only had the lowest levels of both platelets and vWF. Clot analogues containing both Platelets and Plasma had the moderate levels of both platelets and vWF present. An example of a clot analogue closely resembling a clot retrieved from an acute ischemic stroke patient is shown in Figure 3.

Revascularization Results

The three types of clot analogues are associated with different revascularization outcome results (Table 3). The soft clots are associated with the highest Ingestion, FPE and successful recanalization rate, followed by the elastic and stiff clots. The rate of distal embolization increased as the rate of ingestion decreased.

For the soft clots, all of them can be successfully removed within one pass and no distal embolization was observed. Using the DA technique, 80% (4 out of 5) of the clots were ingested (Video S1) with one exception where the catheter tip was corked by the clot. Using the STR+A technique, all the clots can be pulled out without any distal embolization.

For the elastic clot and using the DA technique, only 40% (2 out of 5) of the clots can be ingested and the other 3 clots were corked by the suction catheter and pulled out. During catheter pull, the clot was elongated under the tensional load applied by the vacuum suction and the pressure gradient across the clot. As the clot was moved to the internal carotid artery terminus, a temporary near-to-total obstruction of the flow increased the antegrade pressure gradient and eventually fractured the clot and caused distal embolization (Video S2). In a
similar fashion, the SR+A technique also fractured the clot during device pull, causing distal embolization (Video S3). Distal embolization of clot fragments is the main reason for repeated passes to recanalization.

For the stiff clots and using the DA technique, only 20% (1 out of 5) of the clots can be ingested. During device pull, 80% (4 out of 5) of the clots lost integration with the suction catheter due to the antegrade pressure gradient and resulted in failed revascularization. The integration of the clots to the stent retriever was stronger than the aspiration catheter alone, resulting in a lower distal embolization rate and higher revascularization rate (Table 3).

Table 3: In-vitro thrombectomy results

| Clot type | Ingestion rate | FPE rate | SR rate | Passes to achieve SR | DE rate |
|-----------|----------------|----------|---------|----------------------|---------|
| Soft      |                |          |         |                      |         |
| DA        | 80%            | 100%     | 100%    | 1.0                  | 0       |
| STR+A     | NA             | 100%     | 100%    | 1.0                  | 0       |
| Elastic   |                |          |         |                      |         |
| DA        | 40%            | 100%     | 100%    | 1.0                  | 20%     |
| STR+A     | NA             | 40%      | 100%    | 1.8                  | 60%     |
| Stiff     |                |          |         |                      |         |
| DA        | 20%            | 40%      | 40%     | 1.0                  | 80%     |
| STR+A     | NA             | 60%      | 80%     | 1.3                  | 40%     |

DA: direct aspiration; DE: distal embolization; FPE: first pass effect; SR: successful recanalization; STR+A: Stentriever+Aspiration.
Discussion:

In this study a range of novel *in-vitro* human clot analogues that mimic the gross appearance and histological composition of clots retrieved from acute ischemic stroke patients were created. The composition of the clot analogues was confirmed using the MSB histological stain for the main components and immunohistochemical staining for the identification of Platelets and vWF. Furthermore, a subset of clot analogues were tested in an in-vitro thrombectomy model and demonstrated that revascularization outcome is related to both the composition of the clot and the technique used to retrieve them. The results of this study are important because they 1) prove that human clot analogs that accurately replicate the histological composition of clots retrieved from patients can be created and 2) demonstrate that these clot analogs can be used in an in-vitro thrombectomy setup to compare the performance of different treatment approaches, potentially leading to a clinical benefit for the patients.

The inability of Second Generation thrombectomy aspiration and stentriever devices to dramatically improve the rates of FPE following endovascular treatment of acute ischemic stroke suggests the effect is not specifically device related. An understanding of clot histological characteristics and recanalization outcomes is potentially of great importance in improving device selection and device development. There is a growing awareness about the importance of clot phenotypes, mechanical properties, clot-device interactions and the interactions of clots with the surrounding vessel; factors that influence revascularization rates. However, clinicians largely continue to treat patients using their preferred treatment strategy rather than tailoring their treatment strategy to suit the suspected clot composition. The reason for this is that there have been few clinical studies comparing various thrombectomy techniques in their ability to retrieve different phenotypes of thrombi from...
acute ischemic stroke patients. The histological composition of the clot analogue phenotypes created in this study are in line with the range of clot compositions typically seen in acute ischemic stroke. Clots retrieved from patients can generally be stratified into three main phenotype based on their histological composition; Red Blood Cell-Rich, Fibrin-Rich, and Platelet-Rich. Each of these phenotypes were successfully replicated in this study.

The novel clot analogues described herein used in conjunction with human vascular replicator systems that can accurately replicate the intracranial vasculature, cardiac cycle and intracranial blood pressure may enable the optimization of techniques and treatment strategies. A recent study, using similar human clot analogues in an in-vitro thrombectomy system, also demonstrated that the composition of the clot analogues significantly effects the outcome of the procedure. By assessing the success rate of all of the various thrombectomy devices and techniques in retrieving different phenotypes of clots as described, we can arrive at a better understanding of how to improve the rates of FPE. The use of human clot analogs and accurate in-vitro thrombectomy systems could be a valuable training resource for educating physicians on the potential clinical significance of tailoring their treatment strategy to optimize their chances of achieving complete revascularization. In addition to their use in in-vitro thrombectomy testing, human clot analogs can also be used to investigate the ability of novel diagnostic imaging methods at identifying the composition of the occlusive clot.

For the DA technique, FPE is associated with successful clot ingestion and depends on the clot mechanical properties. Of the 15 clots tested, FPE were achieved for 12 clots and 7 (or 58%) of them were due to successful ingestion. The ingestion rates are 80%, 40%, and 20% for the soft, elastic, and stiff clots. Clots with higher compositions of platelets and fibrin have higher stiffness and friction coefficient, making them difficult to deform into the catheter.
tip and get ingested. For the 8 clots without successful ingestion, 6 (or 75%) of them presented distal embolization and resulted in repeated device passes or failed revascularization. Suction catheters that can generate large suction force to deform the clots and overcome the clot friction to ingest the clots could be beneficial. However, the thrombectomy tests are carried out in a glass phantom and arterial response to suction was not captured. Under suction, the vessel could collapse due to the reduced intraluminal pressure and evacuation of fluid, which was hypothetically related to the more severe vessel injuries using the suction catheters than the stent retrievers. The safety profile of the new-generation large bore suction catheters needs to be further validated.

Comparing the SR+A technique to the DA technique, FPE rate was the same (100%) for the soft clots, lower (40% vs 100%) for the elastic clot, and higher (60% vs 40%) than the DA technique, showing a clear relation with the clot mechanical properties. For both the DA and the SR+A techniques where FPE was not achieved, the failure mechanism was the poor clot/device integration with downstream migration of EA, similar to that found in a whole human brain thrombectomy platform. During clot retrieval by device pull (stent retriever or suction catheter without clot ingestion), the clot/device integration has to fight against the tensional force generated by the device and the antegrade pressure gradient. Compared to the DA technique where the clot was engaged with the suction catheter only at the clot “head” (Video S2), in the SR+A technique, the clot integration was stronger as the clot was grabbed by the stent tines along the clot length (Video S3). This could be the reason of higher FPE and successful revascularization rate for the stiff clot analogues. On the other hand, the clot/stent integration was still weak with multiple passes needed to revascularize. Future stent technologies should enable better clot integration especially for stiff clots.
This study has some limitations. First, the whole blood and platelet donations were not collected from the same patient and the blood phenotypes of each were not available. Second, blood phenotype has been shown to impact coagulation and this method may need to be adjusted slightly for clot phenotype. Finally, the thrombectomy tests are carried out in a glass phantom and arterial response to suction was not captured.
Conclusions:

RBC-rich, Fibrin-rich, and Platelet-rich clot analogues that mimic clots retrieved from Acute Ischemic Stroke Patients were created In-vitro. Differing retrieval outcomes were confirmed using in-vitro thrombectomy testing in a subset of clots. The use of human clot analogs and accurate in-vitro thrombectomy systems could be a valuable training resource for physicians to optimize their chances of achieving complete revascularization for every clot phenotype.

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References:

1. Zaidat OO, Castonguay AC, Linfante I, et al. First Pass Effect. *Stroke* 2018;49:660-666
2. Nikoubashman O, Dekeyzer S, Riabikin A, et al. True First-Pass Effect. *Stroke* 2019;50:2140-2146
3. Todo A, Minaeian A, Sahni R, et al. Incidence and outcome of procedural distal emboli using the Penumbra thrombectomy for acute stroke. *Journal of neurointerventional surgery* 2013;5:135-138
4. Kurre W, Vorlaender K, Aguilar-Pérez M, et al. Frequency and Relevance of Anterior Cerebral Artery Embolism Caused by Mechanical Thrombectomy of Middle Cerebral Artery Occlusion. *American Journal of Neuroradiology* 2013;34:1606
5. Jindal G, Carvalho HDP, Wessell A, et al. Beyond the first pass: revascularization remains critical in stroke thrombectomy. *Journal of NeuroInterventional Surgery* 2019;11:1095
6. Ducroux C, Piotin M, Gory B, et al. First pass effect with contact aspiration and stent retrievers in the Aspiration versus Stent Retriever (ASTER) trial. *Journal of NeuroInterventional Surgery* 2020;12:386
7. Hashimoto T, Hayakawa M, Funatsu N, et al. Histopathologic Analysis of Retrieved Thrombi Associated With Successful Reperfusion After Acute Stroke Thrombectomy. *Stroke* 2016;47:3035-3037
8. Liebeskind DS, Sanossian N, Yong WH, et al. CT and MRI Early Vessel Signs Reflect Clot Composition in Acute Stroke. *Stroke* 2011;42:1237
9. Maegerlein C, Friedrich B, Berndt M, et al. Impact of histological thrombus composition on preinterventional thrombus migration in patients with acute occlusions of the middle cerebral artery. *Interventional Neuroradiology* 2018;24:70-75
10. Saver JL, Goyal M, Bonafe A, et al. Stent-Retriever Thrombectomy after Intravenous t-PA vs. t-PA Alone in Stroke. *New England Journal of Medicine* 2015;372:2285-2295
11. Staessens S, Denorme F, François O, et al. Structural analysis of ischemic stroke thrombi: histological indications for therapy resistance. *Haematologica* 2020;105:498-507
12. Boeckh-Behrens T, Schubert M, Förschler A, et al. The Impact of Histological Clot Composition in Embolic Stroke. *Clinical Neuroradiology* 2016;26:189-197
13. Chueh JY, Wakhloo AK, Hendricks GH, et al. Mechanical Characterization of Thromboemboli in Acute Ischemic Stroke and Laboratory Embolus Analogs. *American Journal of Neuroradiology* 2011;32:1237
14. Preut A, Laughlin M, Jensen H, et al. Novel method for emboli analog formation towards improved stroke retrieval devices. *Journal of Biomechanics* 2018;80:121-128
15. Duffy S, Farrell M, McArdle K, et al. Novel methodology to replicate clot analogs with diverse composition in acute ischemic stroke. *Journal of neurointerventional surgery* 2017;9:486-491
16. Ren M, Lin Z-J, Qian H, et al. Embolic middle cerebral artery occlusion model using thrombin and fibrinogen composed clots in rat. *Journal of Neurosciences Methods* 2012;211:296-304
17. Gunning GM, McArdle K, Mirza M, et al. Clot friction variation with fibrin content; implications for resistance to thrombectomy. *Journal of neurointerventional surgery* 2018;10:34-38
18. Bretzner M, Lopes R, McCarthy R, et al. Texture parameters of R2* maps are correlated with iron concentration and red blood cells count in clot analogs: A 7-T micro-MRI study. *Journal of Neuroradiology* 2019
19. González AV, Buerke B, Görlich D, et al. Clot Analog Attenuation in Non-contrast CT Predicts Histology: an Experimental Study Using Machine Learning. *Translational Stroke Research* 2020;1:1-10
20. Stormont CJ. Blood groups in animals. *J Am Vet Med Assoc* 1982;181:1120-1124
21. Fitzgerald ST, Wang S, Dai D, et al. Platelet-rich clots as identified by Martius Scarlet Blue staining are isodense on NCCT. *Journal of NeuroInterventional Surgery* 2019;11:1145
22. Van Wie BJ. Conceptualization and evaluation of techniques for centrifugal separation of blood cells: optimum process conditions, recycle, and stagewise processing. 1983
23. Staessens S, Fitzgerald S, Andersson T, et al. Histological stroke clot analysis after thrombectomy: Technical aspects and recommendations. *International Journal of Stroke* 2019;1747493019884527

24. Staessens S, Denorme F, Francois O, et al. Dense fibrin, von Willebrand factor and extracellular DNA are specific structural hallmarks of platelet-rich areas in ischemic stroke thrombi. *ISTH 27th Congress of the International Society on Thrombosis and Haemostasis, Location: Melbourne, Australia; 2019*

25. Douglas A, Fitzgerald S, Mereuta OM, et al. Platelet-rich emboli are associated with von Willebrand factor levels and have poorer revascularization outcomes. *Journal of Neurointerventional Surgery* 2019;neurintsurg-2019-015410

26. Mereuta OM, Fitzgerald S, Abbasi M, et al. Abstract WP268: Von Willebrand Factor Expression in Various Subtypes of Acute Ischemic Stroke. *Stroke* 2020;51:AWP268-AWP268

27. Johnson S, Chueh J, Gounis MJ, et al. Mechanical behavior of in vitro blood clots and the implications for acute ischemic stroke treatment. *Journal of neurointerventional surgery* 2019

28. Reddy AS, Liu Y, Cockrum J, et al. Construction of a comprehensive endovascular test bed for research and device development in mechanical thrombectomy in stroke. *Journal of Neurosurgery* 2020;1:1-8

29. Liu Y, Zheng Y, Reddy AS, et al. Analysis of human emboli and thrombectomy forces in large-vessel occlusion stroke. *Journal of Neurosurgery* 2020;1:1-9

30. Ye G, Gao Q, Qi P, et al. The role of diabetes mellitus on the thrombus composition in patients with acute ischemic stroke. *Interventional Neuroradiology;* 0:159101991896940

31. Hashimoto T, Hayakawa M, Funatsu N, et al. Histopathologic analysis of retrieved thrombi associated with successful reperfusion after acute stroke thrombectomy. *Stroke* 2016;47:3035-3037

32. Eng W, Kim M, Pham S, et al. Micromechanics of Blood Clots. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2018;38:A157-A157

33. van der Marel K, Chueh J-Y, Brooks OW, et al. Quantitative assessment of device–clot interaction for stent retriever thrombectomy. *Journal of Neurointerventional surgery* 2016;8:1278-1282

34. Fitzgerald S, Mereuta OM, Doyle KM, et al. Correlation of imaging and histopathology of thrombi in acute ischemic stroke with etiology and outcome. *Journal of neurosurgical sciences* 2019;63:292-300

35. Sporns PB, Jelbmann A, Minnerup J, et al. Histological Clot Composition Is Associated With Preinterventional Clot Migration in Acute Stroke Patients. *Stroke* 2019;50:2065-2071

36. Mohammaden MH, Haussen DC, da Camara CP, et al. Hyperdense vessel sign as a potential guide for the choice of stent retriever versus contact aspiration as first-line thrombectomy strategy. *Journal of NeuroInterventional Surgery* 2020

37. Fitzgerald S, Ryan D, Thornton J, et al. Preclinical evaluation of Millipede 088 intracranial aspiration catheter in cadaver and in vitro thrombectomy models. *Journal of NeuroInterventional Surgery* 2020

38. Liu HC, Abbasi M, Ding YH, et al. Characterizing thrombus with multiple red blood cell compositions by optical coherence tomography attenuation coefficient. *Journal of biophotonics* 2020

39. Liu H-C, Abbasi M, Ding YH, et al. Characterizing blood clots using acoustic radiation force optical coherence elastography and ultrasound shear wave elastography. *Physics in Medicine & Biology* 2020

40. Liu Y, Reddy AS, Cockrum J, et al. Standardized Fabrication Method of Human-Derived Emboli with Histologic and Mechanical Quantification for Stroke Research. *Journal of Stroke and Cerebrovascular Diseases* 2020;29:105205

41. Fitzgerald S, Ryan D, Thornton J, et al. Preclinical evaluation of Millipede 088 intracranial aspiration catheter in cadaver and in vitro thrombectomy models. *Journal of NeuroInterventional Surgery* 2020;neurintsurg-2020-016218
42. Gory B, Bresson D, Kessler I, et al. Histopathologic evaluation of arterial wall response to 5 neurovascular mechanical thrombectomy devices in a swine model. American Journal of Neuroradiology 2013;34:2192-2198

43. Savastano L, Liu Y, Gebrezgiabhier D, et al. O-035 Hybrid human brain model for research in large vessel occlusion stroke. British Medical Journal Publishing Group; 2020
## Appendix

### Table A1: Raw result of revascularization in the benchtop thrombectomy testing platform.

| Clot  | Ingestion? | Direct Aspiration (DA) | Stentriever + Aspiration (STR+A) | DE? |
|-------|------------|-------------------------|----------------------------------|-----|
|       | Pass 1 | Pass 2 | Pass 3 | Pass 1 | Pass 2 | Pass 3 |       |
| Soft  |       |       |       |       |       |       |       |
| #1    | Yes    | SR    | No     | SR    | No     | No     |       |
| #2    | Yes    | SR    | No     | SR    | No     | No     |       |
| #3    | Yes    | SR    | No     | SR    | No     | No     |       |
| #4    | Yes    | SR    | No     | SR    | No     | No     |       |
| #5    | No     | SR    | No     | SR    | No     | No     |       |
| Elastic |       |       |       |       |       |       |       |
| #1    | Yes    | SR    | No     | Fail  | Fail   | SR     | Yes   |
| #2    | Yes    | SR    | No     | SR    | No     | No     |       |
| #3    | No     | SR    | Yes    | Fail  | SR     | Yes    |       |
| #4    | No     | SR    | No     | Fail  | SR     | Yes    |       |
| #5    | No     | SR    | No     | SR    | No     | No     |       |
| Stiff |       |       |       |       |       |       |       |
| #1    | Yes    | SR    | No     | Fail  | Fail   | Fail   | Yes   |
| #2    | No     | Fail  | Fail   | Fail   | Yes    | Fail   | SR    | Yes   |
| #3    | No     | SR    | Yes    | SR    | No     | No     |       |
| #4    | No     | Fail  | Fail   | Fail   | Yes    | SR    | No     |       |
| #5    | No     | Fail  | Fail   | Fail   | Yes    | SR    | No     |       |

SR: successful revascularization; Fail: failed revascularization; DE: distal embolization
Figure 1: Gross Photographs of Human Blood Clot Analogues Pre and Post Fixation. Gross Photographs of Human Blood Clot Analogues of various compositions were taken pre and post fixation in PFA. Clot analogues were created using different ratios (1:5, 1:10, 1:50 and 1:100) of RBC+Buffy Coat and Plasma Only, Platelets Only and Plasma+Platelets.

163x84mm (300 x 300 DPI)
Figure 2: Histological Quantification of MSB stained human clots retrieved from patients and human clot analogues created In-vitro. (A) The histological composition of various 100 clots retrieved from patients was assessed using the MSB stain and grouped according to their dominant component resulting in three main groups; Red Blood Cell-Rich, Fibrin-Rich and Platelet-Rich. (B) The histological composition of various human clot analogues created In-vitro was also assessed using the MSB stain and grouped according to their dominant component resulting in the same three phenotypes; Red Blood Cell-Rich, Fibrin-Rich and Platelet-Rich.
Figure 3: Histology and Immunohistochemical stained images of clots retrieved from an acute ischemic stroke patient and of a human clot analogue. A comparison of the similarity in terms of gross photographs, MSB stain, CD42b (Platelets) and von Willebrand Factor between (A) a clot retrieved from an acute ischemic stroke patient and (B) A human clot analogue (1:50 RBC+Buffy Coat : Platelets) is shown. MSB stain: Red Blood Cells (Yellow), White Blood Cells (Purple), Fibrin (Red), Platelets/Other (Grey), and Collagen (Blue). Platelets (CD42b) and Von Willebrand Factor; Positive staining is Red.
Dear Dr Strother,

Thank you for reviewing our manuscript. Below you will find a point-by-point response to the reviewers’ comments. We hope that by addressing these comments you find our manuscript to be substantially improved. We look forward to hearing your feedback.

Best Regards,
Seán Fitzgerald PhD

REVIEWER'S COMMENTS:

Senior Editor Comments:

1. Please add a paragraph to the Discussion giving your views of how your work adds meaningful information to the extensive literature already existing on this topic.

Response: The authors have added to the first paragraph of the Discussion highlighting the importance of this work. The authors have also added further important relevant information to Paragraph 4 of the discussion.

2. Please delete the last sentence of the Conclusion. The sentence, "These novel human clot analogues will help to advance the field of endovascular device testing and clot imaging research" has no clear meaning. If you wish, replace this with 1 or 2 sentences as to, 1) how this work advances end-vascular device testing, and, 2) how this work advances clot imaging research.

Response: As suggested, this sentence has been removed and replaced with the following:

‘The use of human clot analogs and accurate in-vitro thrombectomy systems could be a valuable training resource for physicians to optimize their chances of achieving complete revascularization for every clot phenotype.’

Reviewer: 1

Comments to the Author

N/A
Reviewer: 2

Comments to the Author

1. Although a large scale verification with the human specimen might be necessary as there should be numerous types of blood clot composition. Meticulous testing of blood clot analogues, along with mechanical thrombectomy shown in this article is useful.

2. The data is fully examined and convincing.

3. I believe an experimental study like this will help the physicians to use the right device in the right situation, which will be beneficial for the patients as well as cost-conscious.

Response: The authors thank Reviewer 2 for their compliments in relation to our manuscript.

Reviewer: 3

Comments to the Author

The authors describe a study in which analogues of human trombi are made, in order to resemble characteristics of thrombi removed from patients with an acute ischemic stroke.

Different types (n=12) of thrombi are made, by changing the amount of RBC + buffy coat, platelets and plasma. These thrombi are thereafter compared to thrombi collected from 100 ischemic stroke patients.

Comparison is made based on gross appearance, as well as histological composition (by MSB stain and immunohistochemistry) in order to differentiate between RBC, WBC, fibrin, platelets, collagen and calcification.

In the second part of the study, they describe results regarding ingestion, first pass effect, and successful recanalization rate from three different artificially made clot types.

Overall, the article is well written and reads easily.

Response: The authors thank Reviewer 3 for their accurate summation of our manuscript.

However, there are some comments I would like to make. I have listed them below.

Several points stand out

- Please clearly describe the aim and hypothesis of the study so that the article can be ‘attached’ to this hypothesis. It will improve the clarity of the study

Response: The authors have added the following hypothesis to the Introduction of the paper:
'The hypothesis of the study is that the diverse range of clots retrieved from AIS patients can be accurately replicated using human blood by mimicking the process by which clots form \textit{in-vivo}.'

- Please improve the methodology. Several items in the result section are not described in the methodology, and statistical methods are currently not included in the manuscript.

Response: The authors have added further detail to several sections of the Methodology and Statistical Analysis Sections as suggested.

- Overall, improve the use of adequate references.

Response: The authors have added several additional references supporting statements made throughout the manuscript.

Introduction:

- The rational of the study is not argued very clearly in my opinion. The authors suggest that because of clot fragmentation and failure to remove the clot, clot composition and treatment strategies should be compared using \textit{in vitro} models.

In the next paragraph, they explain it more clearly. But try to formulate accurately.

Response: As suggested by the Reviewer, the authors have re-written parts of the introduction in an effort to better formulate the rationale of the study.

- A statement is made that blood components vary between species, and that therefore this study should be undertaken. However, no reference about the difference between species is given.

Response: The authors have added an appropriate reference on the variability of blood and blood groups between species to support this statement.

- Please formulate the research question/hypothesis of the study clearly in the end of the introduction. For me, it is not clear what you will be studying in the paper.

Response: The authors have formulated the hypothesis of the study and the rationale at the end of the introduction as suggested by the reviewer.

Methods

- Please use references on why the authors choose for the methodology of human clot analogue creation.

Response: The authors have added a reference in relation to the separation of whole blood into its constituents. The method used to create the human clot analogues is part of the novelty of this study and therefore a reference was not added for the methodology.

- The authors do not clearly specify and explain on what grounds the different clot analogues were chosen.

Response: The authors have added the rationale for the choice of clots analogues included in the study has been added to Paragraph 1 of the ‘Human Clot Analogue Creation’ subsection.
Please specify how embolization events were recorded and scored. Were distal emboli measured in the collected fluid from the different ‘vessels’, or was only visual fragmentation used?

Response: The authors have edited the ‘Thrombectomy Testing in a Benchtop Stroke Platform’ subsection to confirm that only visual fragmentation was used;

‘Distal Embolization was defined as the occurrence of visible fragments of clots being dislodged and migrating distally from the target vessel. The thrombectomy processes were recorded and the failure mechanism, including the presence of Distal Embolization, were confirmed following the procedure.’

The statistical methods are not sufficient for this article. Only one sentence is used on ‘correlations’, however, not one correlation is mentioned in the article. Also, statistical statements are made, but without explanation on what statistics are used. Please provide a power analysis, null hypothesis, p-value, exact statistical methods and change the results and discussion of the article accordingly.

Response: The statistical analysis section has been updated to include the exact statistical methods used and the results and discussion sections of the article have been changed accordingly.

Results

On what criteria are the three different clot types based? Some of the fibrin-rich and platelet-rich seem to have some overlap.

Response: All of the clots, both analogues and clinical samples, are separated into groups based on their dominant component as determined by MSB histological staining. The following sentence has been added to the ‘Histological Processing and Staining’ sub-section of the methodology.

‘Clot phenotype for both the clinical samples and the clot analogs was defined based on the dominant component (%) in each clot as determined by the MSB histological staining.’

In figure 2B, only 11 clots are included instead of 12 described in the methodology.

Response: The reviewer is correct, the histological composition of one clot type had been omitted in error from Figure 2B in the original submission, although the composition was reported in Table 2. The authors have updated Figure 2 to now include all 12 clots described.

The three dominant groups are not pre-defined in the methodology, and therefore seem somewhat artificial and overlap between fibrin-rich and platelet-rich is present.

Response: The three dominant clot phenotypes are now defined at the beginning of the ‘Human Clot Analogue Creation’ subsection in the Methodology. Additionally, as mentioned above, the criteria for determining the clot phenotype is now also stated.

Figure 3: Explain clearly what color the immunohistochemistry positive cells look like, since I only see very little brown positive areas

Response: The authors have added explanation to the figure legend. The positive cells are Red in this instance as the Leica Bond Redmap Kit was used in place of the DAB brown staining kit.
- As mentioned above, the comparisons made in the results section do not seem to be based on statistical methods.

Response: The non-parametric Spearman rho correlation data has been added to the manuscript as requested.

Discussion
- Please update your statistical methods before make statements that clot analogues are the same as clots from AIS patients. The same for revascularization outcomes

Response: The statistical and revascularization outcome methods have been updated as recommended.
- The second part of the discussion (previous studies have demonstrated - of clot composition) seems more something for in the introduction

Response: The authors have removed paragraph 2 as suggested by the reviewer. In addition, the authors have move some information from paragraph 2 to paragraph 3.
- Adequate references are missing throughout the discussion (and manuscript)

Response: The authors have updated references throughout the manuscript to support their statements.
- Suddenly, statements on ingestion rates are made, however, the criteria for these measurements are not described in the methodology.

Response: The authors have added the definitions of Ingestion, First-Pass-Effect, Successful Recanalization and Distal Embolization to the Methods Section of the Manuscript.

Senior Statistical Editor: 1

Comments to Author:

1) Patient Cohort: This section indicates that clots were collected from 100 patients, but does not specify whether a single clot was collected or if multiple clots were collected.

Response: The authors have addressed this comment by editing the Patient Cohort section of the Methods to now read:

‘Clots were collected from 100 patients who underwent mechanical thrombectomy for the treatment of acute ischemic stroke at Mayo Clinic Rochester. Where more than one procedural pass was needed to retrieve the occlusive clot, all fragments of clot were combined for histological analysis.’

2) Statistical Analysis: Please provide a more robust description of the analytic approach used to demonstrate the similarities between the mechanical properties between patient clots and clot analogues, as well as a description of any other statistical analyses utilized.

Response: The authors have added a more robust description of the analytic approach used to demonstrate the similarities between the patient clots and clot analogues as requested by the statistical editor.
3) Figure 2: Figure 2 presents MSB quantification between patient clots and clot analogues, and the Results text concludes on the basis of the data in this figure that the histopathological compositions are ‘similar’ between the two sets of samples. How was ‘similarity’ defined and how were differences assessed between patient clots and clot analogues?

Response: The authors have assessed the similarity between patient clots and clot analogues using the non-parametric Spearman rho correlation.
Novel human acute ischemic stroke blood clot analogues for in-vitro thrombectomy testing

Seán T. Fitzgerald¹,², Yang Liu¹, Daying Dai¹, Oana M. Mereuta¹,²,³, Mehdi Abbasi³, Jorge L. Arturo Larco⁴, Andrew S. Douglas²,³, David F. Kallmes¹, Luis Savastano⁴, Karen M. Doyle²,³, Waleed Brinjikji¹,⁴

¹Department of Radiology, Mayo Clinic, Rochester, MN, USA, ²Department of Physiology, National University of Ireland Galway, Galway, Ireland, ³CÚRAM – SFI Centre for Research in Medical Devices, National University of Ireland Galway, Galway, Ireland, ⁴Department of Neurosurgery, Mayo Clinic, Rochester, MN, USA

Word Count: 4,218-829 Words

Corresponding Author:
Dr Seán Fitzgerald

Address: Department of Physiology, National University of Ireland Galway, Galway, Ireland,

Email: Sean.Fitzgerald@nuigalway.ie

Telephone: +353-91-495397

Fax: 507-255-0706

Twitter: @FitzSeanT
Abstract:

**Background and Purpose:** Previous studies have successfully created blood clot analogues for *In-vitro* endovascular device testing using animal blood of various species. Blood components vary greatly among species, therefore, creating clot analogues from human blood are likely a more accurate representation of thrombi formed in human vasculature.

**Materials and Methods:** Following approval from Mayo Clinic Institutional Review Board, human whole blood and platelet donations were obtained from the Blood Transfusion service. 12 clot analogues were created by combining different ratios of RBC+Buffy coat, Plasma$_2$ and Platelets. Thrombin and CaCl$_2$ were added to stimulate coagulation. Clot composition was assessed using histological and Immunohistochemical staining. To assess the similarities of mechanical properties to patient clots, three types of clot analogues (soft, elastic, and stiff) were selected for in-vitro thrombectomy testing.

**Results:** The range of histopathological compositions produced is representative of clots removed during thrombectomy procedures. The RBC composition ranged from 8.9%-91.4% and Fibrin composition ranged from 3.1%-53.4%. Platelet (CD42b) and von Willebrand Factor (vWF) ranged from 0.5%-47.1% and 1.0%-63.4%, respectively. The soft-clots had the highest first-pass-effect and successful revascularization rates followed by the elastic and stiff clots. Distal embolization events were observed when clot ingestion cannot be achieved, requiring device-pullback. Incidence rate of distal embolization is the highest for the stiff clots due to the weak clot/device integration.

**Conclusions:** RBC-rich, Fibrin-rich, and Platelet-rich and mixed clot analogues that mimic clots retrieved from Acute Ischemic Stroke Patients were created *In-vitro*. Differing retrieval outcomes were confirmed using in-vitro thrombectomy testing in a subset of clots.
**Abbreviations**

DA: Direct Aspiration

DE: Distal Embolization

FFPE: Formalin-fixed paraffin-embedded

FPE: First Pass Effect

MSB: Martius Scarlett Blue

RBCs: Red Blood Cells

STR: Stentriever

SR: Successful Revascularization

WBCs: White Blood Cells
**Introduction:**

In the treatment of acute ischemic stroke, the achievement of complete revascularization from a single mechanical thrombectomy attempt, termed First Pass Effect (FPE), is associated with significantly improved outcomes for patients. Removing the clot in a fragmented manner increases the potential of embolization to new territories, a major contributing factor to poor neurological outcomes due to additional brain infarction.

Despite the advancement in the second generation mechanical thrombectomy devices, the rates of FPE remain low, as low as 29% in the recently reported Aspiration versus Stent Retriever (ASTER) trial.

Previous studies have demonstrated that a wide variety of occlusive clots can cause an LVO and clot composition varies significantly in acute ischemic stroke patients and has been shown to have a significant impact on the success of mechanical thrombectomy procedures. High rates of clot fragmentation and failure to remove the clot in one pass suggests that in order to further advance the success rates of stroke intervention, we must turn our attention to clot composition and compare treatment strategies using in-vitro thrombectomy models of the cerebral vasculature.

Clot composition varies significantly in acute ischemic stroke patients and has been shown to have a significant impact on the success of mechanical thrombectomy procedures. Previous studies have successfully created blood clot analogues for In-vitro testing using animal blood of various species which have significantly advanced our understanding of clot biomechanics and imaging characteristics. However, blood components and blood groups vary greatly amongst species and thus, creating clot analogues with human blood is likely a more accurate representation of thrombi formed in the human vasculature.
We present a novel method of The hypothesis of the study is that the diverse range of clots retrieved from AIS patients can be accurately replicated using human blood by mimicking the process by which clots form in-vivo. The rationale for this study is that, as the success of mechanical thrombectomy procedures is influenced by the composition of the clot, creating human clot analogs that accurately represent the different phenotypes retrieved form patients and testing them in an in-vitro thrombectomy system alters the process by which clots form in-vivo will allow us to compare the performance of different thrombectomy devices and techniques. We will thereby be able to determine the optimum treatment approach for each clot phenotype, thereby. The human clot analogues created can then be inserted in anatomical models of the human vasculature and retrieved using different endovascular strategies. The ability to make accurate clot analogues coupled with the availability of realistic human vasculature replicators will be an invaluable tool to help neurointerventional surgeons test clot analogues of different compositions in various clinical scenarios, thus ultimately moving towards optimizing the chances of achieving the desired First Pass TICI3 outcome in the clinical setting1. To assess the similarities of mechanical properties to patient clots, three types of clot analogues (soft, elastic, and stiff) were selected for in-vitro thrombectomy testing.

Materials and Methods:

Human Clot Analogue Creation

This Study received Institutional Review Board approval from Mayo Clinic Rochester in accordance with the ethical standards of the Declaration of Helsinki. A total of 12 clot analogues types were created as per Table 1. These clots analogues were selected to be representative of the previously identified phenotypes of clots retrieved from AIS patients12.
including; RBC-Rich, Fibrin-Rich, and Platelet-Rich clots\textsuperscript{21}. Clot analogues with an increasing volume of Platelets contract to a greater degree due to the force of platelet contraction, resulting in stiffer clot analogues\textsuperscript{22}.

A human whole blood donation and a human platelet donation from two separate donors were obtained from the Mayo Clinic Blood Transfusion Service. The whole blood was centrifuged at 1,200RPM for 20 minutes at 20°C to separate it into its constituents\textsuperscript{22}. Plasma was harvested by pipetting and the remaining Red Blood Cells and Buffy Coat were mixed together by inverting. A total of 12 clot analogues types were created as per Table 1.\textsuperscript{17} Plasma and Platelets were combined first as per the table and then then 3 µL of Thrombin (1NIH/mL, Roche Diagnostic GmbH, Mannheim, Germany, #T6884) was added to activate platelets for a total of 1-2 minutes whilst continuously mixing. 300 µL of 5% CaCl\textsubscript{2} (Sigma Aldrich, St. Louis, Missouri, United States, #C1016) solution is then added followed by the RBC+Buffy Coat mixture. The tube was then quickly mixed by inversion 5 times and then the clot analogue mixture was drawn into a 3mL syringe. The syringes were spun overnight at 20RPM at room temperature to mimic the dynamic flow conditions of the human vasculature. Clot analogues with an increasing volume of Platelets contract to a greater degree due to the force of platelet contraction, resulting in stiffer clot analogues\textsuperscript{26}.

**Table 1:** Volume of components added to each clot analogue type

| Ratio      | Platelets (µL) | Plasma (µL) | RBC+Buffy Coat (µL) |
|------------|----------------|-------------|---------------------|
| Plasma Only|                |             |                     |
| 1:5        | 0µL            | 2,400µL     | 600µL               |
| 1:10       | 0µL            | 2,700µL     | 300µL               |
| 1:50       | 0µL            | 2,940µL     | 60µL                |
| 1:100      | 0µL            | 2,970µL     | 30µL                |
| Platelets Only|     |             |                     |
| 1:5        | 2,400µL        | 0µL         | 600µL               |
Clots were collected from 100 patients who underwent mechanical thrombectomy for the treatment of acute ischemic stroke at Mayo Clinic Rochester. Where more than one procedural pass was needed to retrieve the occlusive clot, all fragments of clot were combined for histological analysis. The inclusion criteria were; >18 years, having undergone mechanical thrombectomy treatment for acute ischemic stroke and with clot material available for analysis. A waiver of informed consent was granted for the purpose of collecting retrieved clot material from acute ischemic stroke patients for this study.

**Histological Processing and Staining**

Gross photos were taken of each clot and analogue before fixation overnight in 10% phosphate-buffered formalin. All clots and analogues were then processed using a standard tissue processing protocol and embedded in paraffin. The formalin-fixed paraffin-embedded (FFPE) material was cut into 3µm sections. The Martius Scarlett Blue stain is now regarded as the gold-standard for assessing clot composition as it identifies platelet-rich regions of thrombi in addition to RBCs, WBCs and Fibrin. Two representative slides were stained with Martius Scarlett Blue (MSB) to identify the common clot constituents; RBCs, White Blood...
Cells, Fibrin, Platelets/other, Collagen and Calcification as described previously\textsuperscript{21-23}. Clot phenotype for both the clinical samples and the clot analogs was defined based on the dominant component (\%) in each clot as determined by the MSB histological staining.

**Immunohistochemistry**

Platelets and vWF levels are useful additional hallmarks of clot composition\textsuperscript{24-26}. Immunohistochemical staining for platelets (CD42b) and von Willebrand Factor (vWF) was performed on a Leica Bond RX autostainer. Antigen retrieval with Tris-EDTA was performed for platelet staining (anti-CD42b); no antigen retrieval was used for vWF staining. Primary antibody (anti-CD42b; Abcam ab27669, 1:200 dilution, anti-vWF; Dako A-0082, 1:200 dilution); incubation time was 30mins. Negative controls were performed by omission of the primary antibody step. A Leica BOND Polymer Refine Red Detection system that incorporates a post primary antibody, polymer reagent and Fast Red chromogen, and hematoxylin counterstain was used for visualization (Leica Biosystems). Sections were washed in warm soapy water, dehydrated in increasing alcohol gradients, cleared in xylene and mounted with DPX.

**Imaging and Quantification**

Following staining, a representative slide of each stain was scanned at 20x magnification (Motic Easyscan Pro, Motic Digital Pathology). Histologic quantification was performed on the digital slide using Orbit Image Analysis Software (www.Orbit.bio) as described previously. Percentage area of each component (RBC, WBC, Fibrin and
Platelet/other) within the clot was calculated for the histological staining with MSB\textsuperscript{21}. Percentage area of positive IHC staining was calculated separately for CD42b and vWF\textsuperscript{25}.

**Thrombectomy Testing in a Benchtop Stroke Platform**

The mechanical properties of clots vary based on their histological composition; clot analogues with an increasing volume of Platelets contract to a greater degree due to the force of platelet contraction, resulting in stiffer clot analogues\textsuperscript{27}. Three types of clot analogues were selected to represent a variety of patient clots retrieved from thrombectomy: lots analogs that have a high RBC content will typically be softer, more friable clots and clots made from plasma only, will produce clot analogs with a network of thin Fibrin-stands. Three phenotypes of clot analogues with varying compositions of RBCs, Plasma and Platelets were selected to represent prominent phenotypes of clots retrieved from AIS patients during thrombectomy: \textit{soft-Soft} (1:10 RBC + buffy coat: Plasma OnlyRBC:plasma), \textit{Elastic} (1:5 RBC + buffy coat:platelets + plasma), and \textit{Stiff} (1:10 RBC + buffy coat:platelets + plasma).

Thrombectomy testing were carried out on these clots inside a benchtop stroke platform as previously described\textsuperscript{28,29}. Briefly, a cerebrovascular glass model, where the lumen resembles the intracranial internal carotid artery, the anterior cerebral artery, and the middle cerebral arteries, is connected to a customized flow system to deliver flow with physiologically representative flow rate and pressure. Clot analogues measuring 6mm in length were introduced into the flow system and embolized to the M1-M2 bifurcation.
Revascularization was carried out using: 1) the direct aspiration (DA) technique with the Sofia 6F suction-aspiration catheter, and 2) stent retriever with aspiration (SR+A) technique with the Solitaire stent retriever and the Sofia 6F suction-aspiration catheter. For each type of clot analogue and revascularization technique, five clot analogs were made to replicate large vessel occlusion (LVO) stroke. For each LVO case, 3 device passes were attempted before declaring failure. Revascularization result, number of passes, and embolization events were recorded for each test.

Ingestion was defined as complete ingestion of the clot into the catheter, First Pass Effect was defined as complete removal of the clot from the target artery in the first procedural pass, Successful Recanalization (SR) was defined as the complete removal of the clot from the target artery within 3 procedural passes and Distal Embolization was defined as the occurrence of visible fragments of clots being dislodged and migrating distally from the target vessel. The thrombectomy processes were videotaped-recorded and the failure mechanism, including the presence of Distal Embolization, were analyzed and confirmed following the procedure.

Statistical analysis

All statistical correlations were assessed and graphs were generated using GraphPad Prism 8. MSB histological composition was reported as % of the total clot area, positive immunohistochemistry staining (CD42b & vWF) was reported as % of the total clot area. A Shapiro-Wilk test indicated that quantitative variables did not follow a standard normal distribution. The non-parametric Spearman rho correlation was used to assess the similarity between clot analogues and clinical samples.
**Results:**

*Clot Analogue Appearance*

The gross appearance of each of the human clot analogues after clot formation and also post fixation in 10% Neutral buffered formalin is show in Figure 1. Clots analogues that are rich in Red Blood cells clots (e.g. 1:5-10 RBC+Buffy Coat:Plasma Only and Platelets) have a dark red color after creation and a black color post-fixation. Clots that contain high platelet content (e.g. 1:100 RBC+Buffy Coat:Platelets Only) have white platelet-rich regions that are visible both pre and post fixation. Clots that are Fibrin-rich but not platelet-rich have a light red color after creation and a brownish color post-fixation (e.g. 1:50 RBC+Buffy Coat:Plasma Only). The Platelet-rich clots are smaller in clot volume due to the effect of platelet stimulated contraction of the clots. The clot analogues produced have a similar gross appearance to clots extracted from patients during mechanical thrombectomy procedures for the treatment of AIS.
Figure 1: Gross Photographs of Human Blood Clot Analogues Pre and Post Fixation. Gross Photographs of Human Blood Clot Analogues of various compositions were taken pre and post fixation in PFA. Clot analogues were created using different ratios (1:5, 1:10, 1:50 and 1:100) of RBC+Buffy Coat and Plasma Only, Platelets Only and Plasma+Platelets.
**Histological Composition**

The MSB stain was used to assess the histological composition of the clot analogues (Table 2) and of the clots retrieved from AIS patients (Figure 2). Red Blood cell-rich, Fibrin-rich, and Platelet-rich and mixed clot analogues that mimic clots retrieved from acute ischemic stroke patients were created. The range of histopathological compositions of the clot analogues is similar to that of the clinical samples (Figure 2). The addition of a large volume of Red Blood Cells leads to a RBC-Rich clot regardless of the whether platelets and/or plasma were also added (Figure 2). The Red blood cell composition of the clot analogs ranged from 8.9% to 91.4% and the clots retrieved from the patients ranged from <1% to 85%, there was a significant positive correlation between the RBC composition of the analogs and the clinical samples ($r_c=0.755$, $p=0.010^*$). The addition of platelets but not plasma, led to the platelet-rich clots as measured by the MSB stain, platelet composition of the clot analogs ranged from 5.4% to 83.7% whilst the clinical samples ranged from 3% to 88% ($r_c=0.618$, $p=0.048^*$). whilst the addition of plasma only, let to the creation of fibrin-rich clots. The addition of both Plasma and Platelets results in the clots that have both fibrin and platelets present. Fibrin composition of the clot analogs ranged from 3.1% to 53.4% and from 3% to 77% in the clinical samples ($r_c=0.136$, $p=0.694$). White blood cell are typically a minor component of clots and account for an average of 3.5% of clinical clots and levels were about 1% of clot analogs ($r_c=0.311$, $p=0.345$). and Platelets and other ranged from 5.4% to 83.7% (Figure 2). Each of these components is in line with the composition of acute ischemic stroke clots, reported previously in the literature$^{21, 30, 31}$. 
Figure 2: Histological Quantification of MSB stained human clots retrieved from patients and human clot analogues created In-vitro. (A) The histological composition of various 100 clots retrieved from patients was assessed using the MSB stain and grouped according to their dominant component resulting in three main groups; Red Blood Cell-Rich, Fibrin-Rich and Platelet-Rich. (B) The histological composition of various human clot analogues created In-vitro was also assessed using the MSB stain and grouped according to their dominant component resulting in the same three phenotypes; Red Blood Cell-Rich, Fibrin-Rich and Platelet-Rich.
Figure 3: Histology and Immunohistochemical stained images of clots retrieved from an acute ischemic stroke patient and of a human clot analogue. A comparison of the similarity in terms of gross photographs, MSB stain, CD42b (Platelets) and von Willebrand Factor between (A) a clot retrieved from an acute ischemic stroke patient and (B) a human clot analogue (1:50 RBC+Buffy Coat : Platelets) is shown. **MSB stain:** Red Blood Cells (Yellow), White Blood Cells (Purple), Fibrin (Red), Platelets/Other (Grey), and Collagen (Blue). **Platelets (CD42b)** and Von Willebrand Factor; Positive staining is Red.
Table 2: Histological Composition

| Composition Ratio's         | MSB Histological Stain | Immunohistochemistry |         |         |         |         |         |         |         |         |         |
|----------------------------|------------------------|----------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
|                            | Red Blood Cells        | White Blood Cells    | Fibrin  | Platelets and Other | CD42b | Tissue | vWF    | Tissue |
| RBC+BuffyCoat : Plasma Only| 1:5 78.84%             | 0.33%                | 7.61%   | 13.22%             | 2.64% | 97.36% | 1.81%  | 98.19% |
|                            | 1:10 78.02%            | 0.07%                | 13.16%  | 8.74%              | 1.44% | 98.56% | 1.05%  | 98.95% |
|                            | 1:50 28.67%            | 0.15%                | 42.55%  | 28.63%             | 0.46% | 99.54% | 3.27%  | 96.73% |
|                            | 1:100 19.61%           | 0.04%                | 45.73%  | 34.62%             | 3.03% | 96.97% | 1.18%  | 98.82% |
| RBC+BuffyCoat : Platelets Only| 1:5 59.22%             | 1.05%                | 12.59%  | 27.15%             | 30.44%| 69.56% | 19.26% | 80.74% |
|                            | 1:10 30.09%            | 0.91%                | 4.17%   | 64.83%             | 47.11%| 52.89% | 63.43% | 36.57% |
|                            | 1:50 15.33%            | 0.03%                | 5.87%   | 78.77%             | 19.30%| 80.70% | 17.44% | 82.56% |
|                            | 1:100 12.01%           | 0.03%                | 4.29%   | 83.66%             | 25.35%| 74.65% | 34.65% | 65.35% |
| RBC+BuffyCoat : Platelets + Plasma| 1:5 91.42%             | 0.06%                | 3.11%   | 5.41%              | 22.73%| 77.27% | 3.49%  | 96.51% |
|                            | 1:10 77.13%            | 0.19%                | 13.17%  | 9.51%              | 12.31%| 87.69% | 15.47% | 84.53% |
|                            | 1:50 50.51%            | 0.01%                | 19.45%  | 30.03%             | 12.01%| 87.99% | 10.81% | 89.19% |
|                            | 1:100 8.90%            | 0.06%                | 53.39%  | 37.64%             | 11.72%| 88.28% | 2.25%  | 97.75% |
Immunohistochemical Composition

The composition of platelets (CD42b) varied from 0.5% to 47.1% of the total area and the composition of vWF varied from 1.1% to 63.4% of the total area (Table 1). Clot analogues made with platelets only had the largest proportion of both CD42b and vWF present while clot analogues made with plasma only had the lowest levels of both platelets and vWF. Clot analogues containing both Platelets and Plasma had the moderate levels of both platelets and vWF present. An example of a clot analogue closely resembling a clot retrieved from an acute ischemic stroke patient is shown in Figure 3.

Revascularization Results

The three types of clot analogues are associated with different revascularization outcome results (Table 3). The soft clots are associated with the highest Ingestion, FPE and successful recanalization rate, followed by the elastic and stiff clots. The rate of distal embolization increased as the rate of ingestion decreased.

For the soft clots, all of them can be successfully removed within one pass and no distal embolization was observed. Using the DA technique, 80% (4 out of 5) of the clots were ingested (Video S1) with one exception where the catheter tip was corked by the clot. Using the STR+A technique, all the clots can be pulled out without any distal embolization.

For the elastic clot and using the DA technique, only 40% (2 out of 5) of the clots can be ingested and the other 3 clots were corked by the suction catheter and pulled out. During catheter pull, the clot was elongated under the tensional load applied by the vacuum suction and the pressure gradient across the clot. As the clot was moved to the internal carotid artery terminus, a temporary near-to-total obstruction of the flow increased the antegrade pressure gradient and eventually fractured the clot and caused distal embolization (Video S2).
similar fashion, the SR+A technique also fractured the clot during device pull, causing distal embolization (Video S3). Distal embolization of clot fragments is the main reason for repeated passes to recanalization.

For the stiff clots and using the DA technique, only 20% (1 out of 5) of the clots can be ingested. During device pull, 80% (4 out of 5) of the clots lost integration with the suction catheter due to the antegrade pressure gradient and resulted in failed revascularization. The integration of the clots to the stent retriever was stronger than the aspiration catheter alone, resulting in a lower distal embolization rate and higher revascularization rate (Table 3).

Table 3: In-vitro thrombectomy results

| Clot type | Ingestion rate | FPE rate | SR rate | Passes to achieve SR | DE rate |
|-----------|----------------|----------|---------|----------------------|---------|
| Soft      |                |          |         |                      |         |
| DA        | 80%            | 100%     | 100%    | 1.0                  | 0       |
| STR+A     | NA             | 100%     | 100%    | 1.0                  | 0       |
| Elastic   |                |          |         |                      |         |
| DA        | 40%            | 100%     | 100%    | 1.0                  | 20%     |
| STR+A     | NA             | 40%      | 100%    | 1.8                  | 60%     |
| Stiff     |                |          |         |                      |         |
| DA        | 20%            | 40%      | 40%     | 1.0                  | 80%     |
| STR+A     | NA             | 60%      | 80%     | 1.3                  | 40%     |

DA: direct aspiration; DE: distal embolization; FPE: first pass effect; SR: successful recanalization; STR+A: Stentriever+Aspiration.
Discussion:

In this study a range of novel *in-vitro* human clot analogues that mimic the gross appearance and histological composition of clots retrieved from acute ischemic stroke patients were created. The composition of the clot analogues was confirmed using the MSB histological stain for the main components and immunohistochemical staining for the identification of Platelets and vWF. Furthermore, a subset of clot analogues were tested in an in-vitro thrombectomy model and demonstrated that revascularization outcome is related to both the composition of the clot and the technique used to retrieve them. The results of this study are important because they 1) prove that human clot analogs that accurately replicate the histological composition of clots retrieved from patients can be created and 2) demonstrate that these clot analogs can be used in an in-vitro thrombectomy setup to compare the performance of different treatment approaches, potentially leading to a clinical benefit for the patients.

Previous studies have demonstrated that a wide variety of occlusive clots can cause an LVO\textsuperscript{9,26-29}. In early work, clot composition was assessed using Hematoxylin and Eosin staining and clot composition was defined as RBC-Rich (>50%) or Fibrin-Rich. The Martius Scarlett Blue stain is now suggested as the gold-standard for assessing clot composition as it identifies platelet-rich regions of thrombi\textsuperscript{17,20}. Platelet and vWF levels are useful additional hallmarks of clot composition\textsuperscript{21,30-31}. The histological composition of the clot analogue phenotypes created in this study are in line with the range of clot compositions typically seen in acute ischemic stroke\textsuperscript{12}. Clots retrieved from patients can generally be stratified into three main phenotype based on their histological composition: Red Blood Cell Rich, Fibrin Rich, and Platelet Rich. Each of these phenotypes were successfully replicated *in-vitro* this study.
The inability of Second Generation thrombectomy aspiration and stentriever devices to dramatically improve the rates of FPE following endovascular treatment of acute ischemic stroke suggests the effect is not specifically device related. An understanding of clot histological characteristics and recanalization outcomes is potentially of great importance in improving device selection and device development. There is a growing awareness about the importance of clot phenotypes, mechanical properties, clot-device interactions and the interactions of clots with the surrounding vessel; factors that influence revascularization rates. However, clinicians largely continue to treat patients using their preferred treatment strategy rather than tailoring their treatment strategy to suit the suspected clot composition. The reason for this is that there have been few clinical studies comparing various thrombectomy techniques in their ability to retrieve different phenotypes of thrombi from acute ischemic stroke patients. The histological composition of the clot analogue phenotypes created in this study are in line with the range of clot compositions typically seen in acute ischemic stroke. Clots retrieved from patients can generally be stratified into three main phenotype based on their histological composition: Red Blood Cell-Rich, Fibrin-Rich, and Platelet-Rich. Each of these phenotypes were successfully replicated in-vitro this study.

The novel clot analogues described herein used in conjunction with human vascular replicator systems that can accurately replicate the intracranial vasculature, cardiac cycle and intracranial blood pressure may enable the optimization of techniques and treatment strategies. A recent study, using similar human clot analogues in an in-vitro thrombectomy system, also demonstrated that the composition of the clot analogues significantly effects the outcome of the procedure. By assessing the success rate of all of the various thrombectomy
devices and techniques in retrieving different phenotypes of each of the clots as described, we can arrive at a better understanding of how to improve the rates of FPE. The use of human clot analogs and accurate in-vitro thrombectomy systems could be a valuable training resource for educating physicians on the potential clinical significance of tailoring their treatment strategy to optimize their chances of achieving complete revascularization. In addition to their use in in-vitro thrombectomy testing, human clot analogs can also be used to investigate the ability of novel diagnostic imaging methods at identifying the composition of the occlusive clot.

For the DA technique, FPE is associated with successful clot ingestion and depends on the clot mechanical properties. Of the 15 clots tested, FPE were achieved for 12 clots and 7 (or 58%) of them were due to successful ingestion. The ingestion rates are 80%, 40%, and 20% for the soft, elastic, and stiff clots. Clots with higher compositions of platelets and fibrin have higher stiffness and friction coefficient, making them difficult to deform into the catheter tip and get ingested. For the 8 clots without successful ingestion, 6 (or 75%) of them presented distal embolization and resulted in repeated device passes or failed revascularization. Suction catheters that can generate large suction force to deform the clots and overcome the clot friction to ingest the clots could be beneficial. However, the thrombectomy tests are carried out in a glass phantom and arterial response to suction was not captured. Under suction, the vessel could collapse due to the reduced intraluminal pressure and evacuation of fluid, which was hypothetically related to the more severe vessel injuries using the suction catheters than the stent retrievers. The safety profile of the new-generation large bore suction catheters needs to be further validated.
Comparing the SR+A technique to the DA technique, FPE rate was the same (100%) for the soft clots, lower (40% vs 100%) for the elastic clot, and higher (60% vs 40%) than the DA technique, showing a clear relation with the clot mechanical properties. For both the DA and the SR+A techniques where FPE was not achieved, the failure mechanism was the poor clot/device integration with downstream migration of EA, similar to that found in a whole human brain thrombectomy platform. During clot retrieval by device pull (stent retriever or suction catheter without clot ingestion), the clot/device integration has to fight against the tensional force generated by the device and the antegrade pressure gradient. Compared to the DA technique where the clot was engaged with the suction catheter only at the clot “head” (Video S2), in the SR+A technique, the clot integration was stronger as the clot was grabbed by the stent tines along the clot length (Video S3). This could be the reason of higher FPE and successful revascularization rate for the stiff clot analogues. On the other hand, the clot/stent integration was still weak with multiple passes needed to revascularize. Future stent technologies should enable better clot integration especially for stiff clots.

This study has some limitations. First, the whole blood and platelet donations were not collected from the same patient and the blood phenotypes of each were not available. Second, blood phenotype has been shown to impact coagulation and this method may need to be adjusted slightly for clot phenotype. Finally, the thrombectomy tests are carried out in a glass phantom and arterial response to suction was not captured.
Conclusions:

RBC-rich, Fibrin-rich, and Platelet-rich and mixed clot analogues that mimic clots retrieved from Acute Ischemic Stroke Patients were created In-vitro. Differing retrieval outcomes were confirmed using in-vitro thrombectomy testing in a subset of clots. These novel human clot analogues will help to advance the field of endovascular device testing and clot imaging research. The use of human clot analogs and accurate in-vitro thrombectomy systems could be a valuable training resource for physicians to optimize their chances of achieving complete revascularization for every clot phenotype.

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References:

1. Zaidat OO, Castonguay AC, Linfante I, et al. First Pass Effect. *Stroke* 2018;49:660-666
2. Nikoubashman O, Dekeyzer S, Riabikin A, et al. True First-Pass Effect. *Stroke* 2019;50:2140-2146
3. Todo A, Minaeian A, Sahni R, et al. Incidence and outcome of procedural distal emboli using the Penumbra thrombectomy for acute stroke. *Journal of Neurointerventional Surgery* 2013;5:135-138
4. Kurre W, Vorlaender K, Aguilar-Pérez M, et al. Frequency and Relevance of Anterior Cerebral Artery Embolism Caused by Mechanical Thrombectomy of Middle Cerebral Artery Occlusion. *American Journal of Neuroradiology* 2013;34:1606
5. Jindal G, Carvalho HDP, Wessell A, et al. Beyond the first pass: revascularization remains critical in stroke thrombectomy. *Journal of NeuroInterventional Surgery* 2019;11:1095
6. Ducroux C, Piotin M, Gory B, et al. First pass effect with contact aspiration and stent retrievers in the Aspiration versus Stent Retriever (ASTER) trial. *Journal of NeuroInterventional Surgery* 2020;12:386
7. Hashimoto T, Hayakawa M, Funatsu N, et al. Histopathologic Analysis of Retrieved Thrombi Associated With Successful Reperfusion After Acute Stroke Thrombectomy. *Stroke* 2016;47:3035-3037
8. Liebeskind DS, Sanossian N, Yong WH, et al. CT and MRI Early Vessel Signs Reflect Clot Composition in Acute Stroke. *Stroke* 2011;42:1237
9. Maegerlein C, Friedrich B, Berndt M, et al. Impact of histological thrombus composition on preinterventional thrombus migration in patients with acute occlusions of the middle cerebral artery. *Interventional Neuroradiology* 2018;24:70-75
10. Saver JL, Goyal M, Bonafe A, et al. Stent-Retriever Thrombectomy after Intravenous t-PA vs. t-PA Alone in Stroke. *New England Journal of Medicine* 2015;372:2285-2295
11. Staessens S, Denorme F, François O, et al. Structural analysis of ischemic stroke thrombi: histological indications for therapy resistance. *Haematologica* 2020;105:498-507
12. Boeckh-Behrens T, Schubert M, Förschler A, et al. The Impact of Histological Clot Composition in Embolic Stroke. *Clinical Neuroradiology* 2016;26:189-197
13. Chueh JY, Wakhloo AK, Hendricks GH, et al. Mechanical Characterization of Thromboemboli in Acute Ischemic Stroke and Laboratory Embolus Analogs. *American Journal of Neuroradiology* 2011;32:1237
14. Preut A, Laughlin M, Jensen H, et al. Novel method for emboli analog formation towards improved stroke retrieval devices. *Journal of Biomechanics* 2018;80:121-128
15. Duffy S, Farrell M, McArdle K, et al. Novel methodology to replicate clot analogs with diverse composition in acute ischemic stroke. *Journal of neurointerventional surgery* 2017;9:486-491
16. Ren M, Lin Z-J, Qian H, et al. Embolic middle cerebral artery occlusion model using thrombin and fibrinogen composed clots in rat. *Journal of Neuroscience Methods* 2012;211:296-304
17. Gunning GM, McArdle K, Mirza M, et al. Clot friction variation with fibrin content; implications for resistance to thrombectomy. *Journal of neurointerventional surgery* 2018;10:34-38
18. Bretzner M, Lopes R, McCarthy R, et al. Texture parameters of R2* maps are correlated with iron concentration and red blood cells count in clot analogs: A 7-T micro-MRI study. *Journal of Neuroradiology* 2019
19. Gonzalez AV, Buerke B, Görlisch D, et al. Clot Analog Attenuation in Non-contrast CT Predicts Histology: an Experimental Study Using Machine Learning. *Translational Stroke Research* 2020;1-10
20. Stormont CJ. Blood groups in animals. *J Am Vet Med Assoc* 1982;181:1120-1124
21. Fitzgerald ST, Wang S, Dai D, et al. Platelet-rich clots as identified by Martius Scarlet Blue staining are isodense on NCCT. *Journal of NeuroInterventional Surgery* 2019;11:1145
22. Van Wie BJ. Conceptualization and evaluation of techniques for centrifugal separation of blood cells: optimum process conditions, recycle, and stagewise processing. 1983
23. Staessens S, Fitzgerald S, Andersson T, et al. Histological stroke clot analysis after thrombectomy: Technical aspects and recommendations. *International Journal of Stroke* 2019:1747493019884527
24. Staessens S, Denorme F, Françoise O, et al. Dense fibrin, von Willebrand factor and extracellular DNA are specific structural hallmarks of platelet-rich areas in ischemic stroke thrombi. *ISTH 27th Congress of the International Society on Thrombosis and Haemostasis, Location: Melbourne, Australia; 2019*
25. Douglas A, Fitzgerald S, Mereuta OM, et al. Platelet-rich emboli are associated with von Willebrand factor levels and have poorer revascularization outcomes. *Journal of NeuroInterventional Surgery* 2019:neurintsurg-2019-015410
26. Mereuta OM, Fitzgerald S, Abbasi M, et al. Abstract WP268: Von Willebrand Factor Expression in Various Subtypes of Acute Ischemic Stroke. *Stroke* 2020;51:AWP268-AWP268
27. Johnson S, Chueh J, Gounis MJ, et al. Mechanical behavior of in vitro blood clots and the implications for acute ischemic stroke treatment. *Journal of neurointerventional surgery* 2019
28. Reddy AS, Liu Y, Cockrum J, et al. Construction of a comprehensive endovascular test bed for research and device development in mechanical thrombectomy in stroke. *Journal of Neurosurgery* 2020;1:1-8
29. Liu Y, Zheng Y, Reddy AS, et al. Analysis of human emboli and thrombectomy forces in large-vessel occlusion stroke. *Journal of Neurosurgery* 2020;1:1-9
30. Ye G, Gao Q, Qi P, et al. The role of diabetes mellitus on the thrombus composition in patients with acute ischemic stroke. *Interventional Neuroradiology* 0:1591019919896940
31. Hashimoto T, Hayakawa M, Funatsu N, et al. Histopathologic analysis of retrieved thrombi associated with successful reperfusion after acute stroke thrombectomy. *Stroke* 2016;47:3035-3037
32. Eng W, Kim M, Pham S, et al. Micromechanics of Blood Clots. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2018;38:A157-A157
33. van der Marel K, Chueh J-Y, Brooks OW, et al. Quantitative assessment of device–clot interaction for stent retriever thrombectomy. *Journal of neurointerventional surgery* 2016;8:1278-1282
34. Fitzgerald S, Mereuta OM, Doyle KM, et al. Correlation of imaging and histopathology of thrombi in acute ischemic stroke with etiology and outcome. *Journal of neurosurgical sciences* 2019;63:292-300
35. Sporns PB, Jeibmann A, Minnerup J, et al. Histological Clot Composition Is Associated With Preinterventional Clot Migration in Acute Stroke Patients. *Stroke* 2019;50:2065-2071
36. Mohammaden MH, Haussen DC, da Camara CP, et al. Hyperdense vessel sign as a potential guide for the choice of stent retriever versus contact aspiration as first-line thrombectomy strategy. *Journal of NeuroInterventional Surgery* 2020
37. Fitzgerald S, Ryan D, Thornton J, et al. Preclinical evaluation of Millipede 088 intracranial aspiration catheter in cadaver and in vitro thrombectomy models. *Journal of NeuroInterventional Surgery* 2020
38. Liu HC, Abbasi M, Ding YH, et al. Characterizing thrombus with multiple red blood cell compositions by optical coherence tomography attenuation coefficient. *Journal of biophotonics* 2020
39. Liu H-C, Abbasi M, Ding YH, et al. Characterizing blood clots using acoustic radiation force optical coherence elastography and ultrasound shear wave elastography. *Physics in Medicine & Biology* 2020
40. Liu Y, Reddy AS, Cockrum J, et al. Standardized Fabrication Method of Human-Derived Emboli with Histologic and Mechanical Quantification for Stroke Research. *Journal of Stroke and Cerebrovascular Diseases* 2020;29:105205
41. Fitzgerald S, Ryan D, Thornton J, et al. Preclinical evaluation of Millipede 088 intracranial aspiration catheter in cadaver and in vitro thrombectomy models. *Journal of NeuroInterventional Surgery* 2020:neurintsurg-2020-016218
42. Gory B, Bresson D, Kessler I, et al. Histopathologic evaluation of arterial wall response to 5 neurovascular mechanical thrombectomy devices in a swine model. *American Journal of Neuroradiology* 2013;34:2192-2198

43. Savastano L, Liu Y, Gebrezgiabhier D, et al. O-035 Hybrid human brain model for research in large vessel occlusion stroke. British Medical Journal Publishing Group; 2020
**Appendix**

**Table A1: Raw result of revascularization in the benchtop thrombectomy testing platform.**

| Clot   | Direct Aspiration (DA) | Stentriever + Aspiration (STR+A) |
|--------|------------------------|----------------------------------|
|        | Ingestion?  | Pass 1 | Pass 2 | Pass 3 | DE? | Pass 1 | Pass 2 | Pass 3 | DE? |
| Soft   |            |        |        |        |     |        |        |        |     |
| #1     | Yes        | SR     | No     | SR     | No  |
| #2     | Yes        | SR     | No     | SR     | No  |
| #3     | Yes        | SR     | No     | SR     | No  |
| #4     | Yes        | SR     | No     | SR     | No  |
| #5     | No         | SR     | No     | SR     | No  |
| Elastic|            |        |        |        |     |        |        |        |     |
| #1     | Yes        | SR     | No     | Fail   | Fail | SR     | Yes   |
| #2     | Yes        | SR     | No     | SR     | No  |
| #3     | No         | SR     | Yes    | Fail   | SR   | Yes   |
| #4     | No         | SR     | No     | Fail   | SR   | Yes   |
| #5     | No         | SR     | No     | SR     | No  |
| Stiff  |            |        |        |        |     |        |        |        |     |
| #1     | Yes        | SR     | No     | Fail   | Fail | Fail   | Yes   |
| #2     | No         | Fail   | Fail   | Fail   | Yes | Fail   | SR    | Yes   |
| #3     | No         | SR     | Yes    | SR     | No  |
| #4     | No         | Fail   | Fail   | Fail   | Yes | SR     | No    |
| #5     | No         | Fail   | Fail   | Fail   | Yes | SR     | No    |

SR: successful revascularization; Fail: failed revascularization; DE: distal embolization