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Shining a light on platelet activation in COVID-19

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Coagulopathy and platelet hyperactivity are hallmarks of coronavirus disease 2019 (COVID-19) and contribute to an increased thrombotic burden observed in many COVID-19 patients.¹ Anticoagulation and/or antiplatelet drugs are actively pursued in clinical trials aimed at improving outcomes in COVID-19 patients. However, while a consensus exists on the benefit of early anticoagulation, no consensus has been reached for antiplatelet drugs. A recent study, published in *Nature Communications*, described a novel technique for imaging and quantifying platelet aggregates in the circulation of COVID-19 patients longitudinally.² This technique could potentially help stratify patients who would benefit the most from antiplatelet drugs in COVID-19. Moreover, this high-throughput microscopy technique could have applications in other thrombotic disorders in which platelets play a key role in thrombotic complications.

1 | ANTICOAGULATION IN COVID-19: STATE OF THE ART

Thromboembolic complications are more commonly observed in COVID-19 patients compared to other acute medical illnesses and respiratory infections.^{3,4} Importantly, widespread pulmonary thrombosis, as found in COVID-19 autopsies, imply this coagulopathy also contributes to respiratory failure.⁵⁻⁷ In addition, COVID-19 coagulopathy is independently associated with critical illness and all-cause mortality.^{8,9} With these observations in mind, several trials combined forces to evaluate therapeutic-dose versus prophylactic-dose heparin, in hospitalized COVID-19 patients (ACTIV-4a, ATTACC, and

REMAP-CAP).^{10,11} These trials were halted in December 2020 for two distinct and intriguing reasons. In critical care patients, the trials were stopped due to futility, attributed to a high risk for bleeding complications.¹¹ Conversely, in moderately ill COVID-19 patients, the trials were halted due to superiority, demonstrating robust benefits of therapeutic-dose heparin.¹⁰ These results highlight the complexity of COVID-19-associated coagulopathy and the importance of starting therapeutic heparin early in the disease process, before patients become critically ill.

Remarkably, many of the COVID-19 autopsy studies that found evidence for micro- and macro-thrombotic events were performed on patients that were on prophylactic or therapeutic anticoagulant treatment.⁴⁻⁷ Likewise, the clinical trials that demonstrated superiority for anticoagulant treatment, still observed significant thrombotic events.^{10,11} This implies there is potential for additional adjunctive antithrombotic treatments, with antiplatelet drugs as the prime candidates.

2 | PLATELETS IN COVID-19: A DOUBLE-EDGED SWORD

While classically known for their hemostatic function, platelets are also gaining recognition as an integral part of the immune system, teaming up with innate immune cells such as neutrophils and macrophages to actively trap and kill pathogens.¹² Platelets bridge the immune system and thrombosis via activation and release of hemostatic and inflammatory mediators. The dark side of this immune phenotype of platelets, however, is the collateral damage associated with systemic platelet activation. Indeed, the interplay among

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platelets, coagulation, and the endothelium can result in thrombotic complications, as often observed in viral and bacterial infections.¹³ Likewise, it has been hypothesized that platelets contribute to thrombosis in COVID-19 patients. Platelet-rich thrombi were readily observed in COVID-19 patients,^{5,14} indicating they actively participate in COVID-19-associated thrombosis. Furthermore, we and others have found that COVID-19 is associated with a hyperactive platelet phenotype, characterized by increased platelet activation, platelet extracellular vesicle release, and platelet-leukocyte and platelet-endothelial cell interactions.¹⁵⁻²⁰ Interestingly, several studies have reported a link between platelet activation markers and outcomes in COVID-19 patients highlighting potential for antiplatelet drugs in COVID-19.^{15,18,21,22} Antiplatelet therapy might have beneficial effects in severe COVID-19 through several mechanisms, including inhibition of platelet aggregation as well as reduction of platelet-derived inflammation.^{23,24} *In vitro*, we and others have shown that aspirin reduces the hyperactive platelet phenotype in platelets isolated from COVID-19 patients.^{20,25} Similarly, Barrett et al. found that P2Y₁₂ inhibition was able to reduce platelet-mediated endothelial cell activation in COVID-19 patients.¹⁸

However, the real-world translation of these promising findings has turned out to be not as straightforward as initially hoped. The RECOVERY trial evaluated the addition of aspirin to standard care (i.e., therapeutic heparin) and found no improvement with regard to reductions in the risk of progressing to invasive mechanical ventilation or death.²⁶ Nevertheless, aspirin use was associated with a slight yet significant reduction in thrombotic events, again corroborating the role of platelets in COVID-19-associated thrombosis. Unfortunately, a similar increase in bleeding events was found, potentially contributing to the net zero effect of aspirin on outcomes.²⁶ Despite these discouraging first results, there was the potential for other antiplatelet drugs such as P2Y₁₂ inhibitors to be more effective due to their potent platelet-inhibiting properties and distinct anti-inflammatory effects.²⁷ Nonetheless, the ACTIV-4a clinical trial again showed no effect for the combination of therapeutic dose heparin plus a P2Y₁₂ inhibitor in non-critically ill COVID-19 patients.²⁸ Intriguingly, heparin plus a P2Y₁₂ inhibitor did not reduce major thrombotic events compared to heparin alone. While discouraging, it is important to note that these trials assessed the effect of antiplatelet drugs on top of therapeutically dosed heparin, thereby making it difficult to detect (additional) benefit.²⁹

It was postulated that other drugs, more specifically targeting the thromboinflammatory effects of platelets, have a better chance at improving outcomes. However, both aspirin and P2Y₁₂ inhibitors have well-characterized anti-inflammatory effects indicating altering the timing of antiplatelet treatment, rather than type, could be clinically beneficial. Several observational retrospective studies have indeed found an association with pre-hospital antiplatelet drugs and improved outcomes in hospitalized COVID-19 patients.³⁰⁻³² Given the robust transcriptional differences in COVID-19 patient platelets,^{20,22} observed irrespective of disease severity and time of hospital admission, it is reasonable to assume irreversible platelet changes have already taken effect before antiplatelet treatment was

started in these trials. In addition to knowing when to treat patients with platelet inhibitors, it is conceivable not every COVID-19 patient would benefit the same from antiplatelet drugs. In light of this, the novel method presented by Nishikawa et al.,² which uses fluorescent single-cell profiling of circulating platelet aggregates, has the potential to become a valuable tool to stratify COVID-19 patients in terms of platelet activation status.

3 | FLUORESCENT SINGLE-CELL PLATELET PROFILING IN COVID-19 PATIENTS

The ability to visualize platelet-platelet interactions and platelet-leukocyte aggregates in a meaningful approach with high-throughput processing is a significant limitation with current technology. Flow cytometry methodologies allow for robust characterization of platelet-leukocyte interactions using small quantities of blood. However, flow cytometry has limitations in distinguishing platelet-platelet interactions from single platelets due to limits on spatial resolution. On the other hand, optical microscopy is well equipped to visualize individual platelet-platelet interactions as microscopy allows the user to discriminate between single platelets and platelet-platelet aggregates. However, microscopic analysis is labor intensive and tedious to statistically examine enough aggregates to infer meaningful conclusions. To overcome these limitations, Nishikawa et al. merged the best of both worlds and developed a microfluidics system combined with frequency-division-multiplex (FDM) microscopy to examine platelet-platelet aggregates through massive single-cell image-based profiling.² FDM microscopy allows for high spatial and temporal resolution permitting image acquisition that was triggered when platelets were detected by a fluorescence signal, allowing the microscope to focus on platelet events and by-passing non-platelet events. In addition to high-resolution imaging, this novel approach allows for the use of small volumes of blood. A relatively easy sampling process allowed them to image 250,000 events including single platelets, platelet-platelet aggregates, and platelet-leukocyte aggregates per sample. Using this technique, excessive platelet aggregates were observed in nearly 90% of all analyzed COVID-19 patients, while these events were rare in healthy subjects. More importantly, more severe COVID-19 patients had a higher concentration of platelet aggregates independent from D-dimer levels suggesting platelet-platelet aggregates are a more sensitive measure of disease severity. However, more studies are needed to validate these findings. While the study offers a novel technique for examining platelet-platelet aggregates, the use of specialized equipment may preclude the widespread utilization of this procedure. Finally, a limitation of the current study was the daily measurement of platelet-platelet aggregates with only the highest daily platelet-platelet aggregate levels correlating with clinical outcomes. Therefore, it remains to be evaluated how easy to use this technique will be in a clinical setting.

4 | FUTURE DIRECTIONS: APPLICATIONS BEYOND COVID-19

While this novel technique will need fine-tuning and validation by other research groups, it has the potential to become a valuable additional tool to study platelet hyperactivation in patients. As platelets are key drivers of many thrombotic and inflammatory disorders, this technique could have applications that go beyond COVID-19 such as sepsis, thrombotic thrombocytopenic purpura, and ischemic stroke. While the emphasis of the current study was on COVID-19 patients, it will be important to consider this technique in other diseases in which antiplatelet drugs might not benefit all patients equally. In particular, this technique could help stratify patients who are most likely to benefit from antiplatelet therapies in diseases in which a risk for bleeding limits the efficacy of antiplatelet drugs—in the setting of stroke, for example, in which cerebral bleedings are a life-threatening complication of antiplatelet drugs.^{33–35} While single or dual antiplatelet treatments have shown great potential in stroke prevention,³⁵ variable results have been reported for their use in the setting of acute ischemic stroke treatment.³⁶ Studies performed in animal models have found platelets contribute to ischemic stroke by participating in occlusive thrombus formation and embolization, but also by acting downstream of the occlusive thrombus, contributing to reperfusion injury and the no-reflow phenomenon.³⁷ It still a topic of debate whether similar phenomena contribute to human stroke pathophysiology; however, robust studies on platelet activation markers in the acute setting of stroke are lacking and their contribution to stroke outcomes has never thoroughly been investigated. Future studies should analyze whether circulating platelet aggregates are observed in stroke patients and whether these are able predict long-term stroke outcomes.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

F.D., A.A., and R.A.C. wrote the paper.

INFORMED CONSENT

On behalf of all the authors of the publication “Shining a light on platelet activation in COVID-19”, I declare that all the authors have read, critically reviewed the paper, and agree to its publication.

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