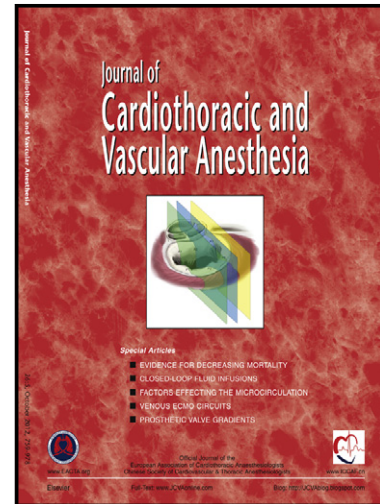# Author's Accepted Manuscript

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Introduction

Significant postoperative bleeding is a common risk of cardiac surgery with approximately 3.5% of patients requiring surgical re-exploration.[1] Re-exploration is associated with adverse outcomes including infections, ischemia, and increased 30-day mortality.[2] Similar adverse outcomes are related to erythrocyte transfusions associated with cardiac surgery,[3] in addition to the immunologic and administrative hazards of transfusion.[4] These risks are important because the majority of patients undergoing cardiac surgery receive a blood transfusion despite the lack of evidence to support liberal transfusion strategies.[5] The frequency and significance of bleeding following cardiac surgery warrants investigation of the hematologic changes throughout the procedure. This review focuses on the (patho)physiology of platelet-derived microvesicles in the setting of cardiovascular surgery, a developing area in our understanding of the control of coagulation.

Microvesicle physiology

Microvesicles, formerly called microparticles,[6,7] are released from the cell membrane of a range of cells, including monocytes, erythrocytes and endothelial cells, during activation or apoptosis.[8,9] They are 10-200 nm in diameter and retain surface antigens specific to their parent cell. Platelet microvesicles (PMV) are the most abundant in normal plasma.[7,10] PMV have an important role in the initiation and propagation of coagulation, providing a key focus for hematologic research to improve the safety of cardiac surgery. Their discovery occurred when the clotting time of blood was prolonged following high-speed centrifugation during which PMV became separated from platelet-dense serum, suggesting that PMV enhance coagulation.[11] Further research using centrifugation and electron microscopy revealed that activated platelets produced a phospholipid-rich 'platelet dust' which had a role in thrombin generation.[12]

Production of PMV

Following endothelial injury, inflammatory mediators including glycoprotein Ib/IX and endothelial von Willebrand factor (vWf) trigger a rise in cytosolic calcium which stimulates calpain to trigger platelet activation and the release of adenosine diphosphate (ADP), thrombin, tissue factor (TF) and thromboxane A_2.[9] (Figure 1).

In the non-activated state the phospholipid bi-layer of the platelet membrane is held in asymmetry as a result of the enzyme transporters flippase and aminophospholipid translocase.[13] A rise in cytosolic calcium following platelet activation has a key role in the production of PMV through its action on these enzymes (Figure 2). Flippase and aminophospholipid translocase are inhibited, disabling them from holding phosphatidylserine (PS) and phosphatidylethanolamine on the inner membrane layer,[14] while activation of floppases and lipid scramblase causes rapid outward translocation of PS and loss of membrane asymmetry.[15,16] The movement of PS to the outer surface of the platelet membrane activates factor X and prothrombin, thus propagating the coagulation cascade and the formation of PMV.[17,18]

In addition to the loss of membrane asymmetry, activation of gelsolin and calpain causes is alteration of the platelet actin cytoskeleton and a change in platelet shape, which in itself produces PMV.[19,20] However, PMV are still produced when calpain is inhibited.[21] There are consequentially multiple pathways to PMV production, and disabling one pathway may still allow PMV to be shed.

Procoagulant activity

The inability to produce lipid scramblase in Scott syndrome results in platelets that are unable to lose membrane asymmetry to allow surface expression of PS and form microvesicles.[9] Platelets have rapid PS expression, allowing prompt promotion of coagulation at the sites of endothelial injury;[18] the removal of PS from the PMV surface decreases the rate of thrombin production,[22] providing evidence that the production of PMV

is critical to physiological coagulation. The phenotype of a microvesicle alters its function; increasing expression of PS and TF enhances thrombin generation.[22] Some PMV express anti-coagulant receptors, suggesting there may be a role for PMV in regulating hemostasis.[23] Procoagulant receptors are more densely expressed on the surface of PMV than the parent platelet by up to 100-fold.[24] Increased concentrations of circulating PMV promote platelet aggregation, leading to thrombus formation.[25] PS expression on the outer surface of the microvesicle encourages coagulation due to its ability to bind and stimulate several coagulation factors through the interaction with their gamma-carboxyglutamic acid domains. These factors include factors IIa, Va, VIII, IXa (Figure 3).[26] TF expressed on the surface of PMV initiates coagulation by activating factor VII to recruit platelets and initiate the pathway to thrombus formation in the response to vascular injury.[27] However increased TF expression does not always correlate with increased coagulation, suggesting that other factors are similarly important.[22,28]Integrin $\alpha_{11b}\beta_3$, another receptor expressed on the outer PMV surface, has a role in binding PMV to fibrinogen to cause platelet adhesion.[29]

Microvesicles taken from the pericardial cavity of patients undergoing cardiopulmonary bypass promote thrombin generation.[30] Berckmans *et al* demonstrated that the introduction of microvesicles *in vitro* to plasma results in thrombin generation.[26] Interestingly, they proposed that small amounts of thrombin generate activated protein C (APC), which can have anticoagulant properties by inactivating factors Va and VIIIa.[26] PMV also support APC generation, which leads to a hypothesis that PMV have a role in homeostasis in thrombosis by possessing both anti- and pro-coagulant triggering mechanisms.[23,31]

Clearance

The first report investigating the half-life of transfused PMV *in vivo* demonstrated a plasma half-life of under six hours in 11 patients with thrombocytopenia awaiting platelet transfusion.[32] This is faster than the rate of circulating platelet clearance. Microvesicles released following dobutamine stress echocardiography are cleared from the circulation

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within an hour.[33] These studies suggest that microvesicles exert their actions in a very short

time-frame and that there exists separate pathways for microvesicle and platelet clearance.

There are significantly higher numbers of circulating microvesicles in splenectomised mice

versus non-splenectomised mice, suggesting that the spleen is the site of microvesicle

clearance.[34] Following opsonisation of microvesicles, macrophages can then phagocytose

particles up to 2µm in size.[35] The process is very rapid, with maximum uptake occurring at

one hour.

Platelet microvesicles decline more gradually in thawed fresh frozen plasma, by 50% over

five days. The resulting decline in procoagulant activity was 29%, indicating that PMV

numbers decrease *in vitro* and contribute to *in vivo* coagulation.[10] The same study also

demonstrated that removal of PMV by filtration results in reduced thrombin generation, and

that restoration of PMV into the infusion increased the ability for coagulation. The cause of

PMV decline *in vitro* is only speculated; Matijevic *et al* suggests that extacellular

phospholipases may degrade the microvesicles to sub-detectable volumes or that

microvesicle coupling to other microvesicles or platelets occurs, forming larger aggregates.[36]

The decline in microvesicle numbers may have been a result of proteases degrading

antibodies on the vesicle surface over time therefore creating an apparent decline in

numbers.

Laboratory tests and PMV

The standard method for monitoring coagulation during CPB is the Activated Clotting Time

(ACT) of whole blood. However, this has poor reproducibility, with variation between

activators and between devices, and has no correlation with heparin concentration.[37,38] ACT

has no role in evaluation of platelet or PMV function. The activated partial thromboplastin

time and the prothrombin time are used in laboratory practice to assess the intrinsic and

extrinsic coagulation pathways respectively, but since they are performed on cell-free

plasma neither of these tests is sensitive or specific enough for the complete assessment of

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either perioperative coagulation status or PMV activity. Tests of the visco-elastic properties of whole blood, such as thromboelastography, allow more precise evaluation of perioperative coagulopathy, however thromboelastography does not seem to be effective at analysing PMV populations.[39] The standard method for evaluating PMV is flow cytometry (see below), but this is not currently available as a point-of-care test.

Pharmaceutical influences

Antiplatelet agents

Antiplatelet drug use before cardiac surgery increases bleeding complications.[40,41] Aspirin inhibits cyclooxygenase-1 derived thromboxane A_2 in platelets, limiting cytosolic calcium increases to reduce aggregation. Aspirin ingestion for seven days does not affect either the number or surface expressions of PMV.[42] Clopidogrel acts through the inhibition of ADP-induced aggregation. ADP produced from activated platelets triggers G-protein coupled receptors to increase cytosolic calcium to cause PMV production.[43] Clopidogrel use in the 24 hours prior to cardiac surgery has an odds ratio of 2.4 for excessive bleeding compared to patients not receiving clopidogrel.[40] The plasma concentration of clopidogrel is inversely proportional to the number of PMV in patients with stable coronary artery disease, suggesting clopidogrel reduces the production of PMV.[44] Similarly, plasma endothelial microvesicle numbers are reduced after administration of clopidogrel.[45] However, one study found that anti-platelet drugs did not have an effect on populations of circulating PMV.[46] These conflicting results warrant further investigation into the effect of antiplatelet agents on PMV populations.

Anticoagulants

Heparin inhibits thrombin via anti-thrombin III, but does not affect surface-bound thrombin.[47] Muriithi *et al* found that a delayed heparin-induced impairment of platelet aggregation occurs in patients prior to CPB, which may suggest that platelet defects previously thought to be solely the result of passage through the bypass circuit may result from interaction with

heparin.[48] Although Muriithi's study did not investigate PMV, their formation occurs simultaneously with platelet activation and therefore heparinisation may result in decreased PMV numbers.

Anesthetics and other drugs

Currently no studies have investigated the effect of propofol or volatile anesthetics on PMV populations, however propofol does inhibit both intra-operative and post-operative platelet aggregation.[49,50] Given the hemodynamic effects of anesthetic drugs, changes in shear stress within the circulation may be expected to modify platelet and PMV behaviour, but there are no published data on how this may affect PMV. Statins have no effect on PMV numbers.[44,46]

Intravenous fluids

A variety of crystalloid and colloid fluids, and blood components, are administered during surgery and CPB. Hemodilution reduces blood viscosity, allowing potentially faster blood flow and increased shear stresses within the cardiovascular system. However, there are no published data on how this might affect PMV.

Microvesicles in health and disease

Females have more circulating PMV than males, although this difference is reduced following the menopause.[7] Low or normal levels of PMV occur in pre-eclampsia, while systemic lupus erythematosus, paroxysmal nocturnal hemoglobinuria, myeloproliferative diseases, venous thromboembolism, diabetes mellitus, acute coronary syndrome, and chronic renal failure result in raised PMV numbers.[51]

PMV in cardiac surgery

Cardiopulmonary bypass

The process of CPB induces hematologic changes via various pathways, including the passage of blood through the circuit causing activation of coagulation factors, adhesion of blood components to the circuit, and hemodilution as the priming solution mixes with the patient's blood. The effect of CPB on coagulation is significant; excessive bleeding is more common in patients undergoing on-pump cardiac surgery than in those undergoing off-pump cardiac surgery.[52] However,circulating fresh blood through a miniaturized CPB circuit does not stimulate PMV production,[53] suggesting that surgical trauma is more of a stimulus to PMV production than CPB.

Shear stress

Cardiopulmonary bypass is usually performed with roller pumps, rather than centrifugal pumps, despite the generation of shear forces that can cause hemolysis, and spallation of the pump head tubing.[54,55] There is no evidence favoring either type of pump in terms of hematologic or coagulation parameters, postoperative blood loss, blood transfusion, neurologic outcome, or mortality,[41,56-58] since the effects of antifibrinolytic drugs and mild hypothermia probably overshadow any small differences between pump types.[41] However, damage to platelets and red cells, and the inflammatory response, may be worse with prolonged CPB using roller pumps.[56,59,60] Shear stress causes the platelets to bind vWf,[61] the surrounding hydrodynamic forces inducing change in platelet shape,[62] and hence the production of PMV, but there are no published data on the effect of pump type on PMV.

Adhesion

Despite complement activation during CPB, PMV numbers decrease even after hemodilution is accounted for.[63] These findings suggest that adhesion to the circuit,[64,65] or recruitment to thrombus formation within the surgical field, decreases the PMV population faster than they are being produced. The thrombogenic surface of the CPB circuit encourages platelet

activation and the production of PMV,[66] though this effect is reduced with bio-compatible coatings in the CPB circuit.[67] Fibrin and fibrinogen adhere to the surface, causing thrombin to be deposited, which then binds platelet receptors causing their deposition on the circuit surface.[62] PMV bind to collagen type I, fibrinogen, vWf, and also to immobilized platelets already bound to thrombogenic surfaces. Due to the sub-micron size of PMV, they may be able to withstand extreme shear stress, remaining in position to encourage further thrombus formation on extra-corporeal surfaces.[68]

Hemodilution

The prime fluid mixes with circulating blood, diluting coagulation factors both during bypass and in the immediate post-bypass period.[69] As mean arterial pressure is low during CPB there is an increased risk of thrombotic events during this time, counteracted by the reduction in pro-coagulant substances, cells and PMV by hemodilution. The risk of adverse hematologic events occurs post-operatively when these key players in the coagulation cascade have not recovered to pre-bypass levels, leading to a coagulopathy.

Cell salvage

Mechanical cell salvage removes the majority of platelets and virtually all PMV,[70] since PMV and coagulation factors are removed in the washing process. Despite this reduction, the adverse event incidence following autotransfusion is 0.027% in contrast to 0.14% with allogeneic blood transfusions.[71] However, postoperative platelet counts two hours after CPB are the same between control and cell-salvaged patients, although postoperative blood loss was less in patients who had received cell-salvaged blood.[72] Further work is required to elucidate the pattern of PMV activity during cardiac surgery and the potential impact on perioperative coagulation.

Surgical trauma

Surgical trauma causes endothelial and soft tissue injury which leads to PMV formation.[51,73-75] The result is platelet recruitment, and subsequent thrombus formation. Surgical trauma increases microvesicle production in cardiac surgery in addition to the effect of CPB, since pericardial blood contains higher concentrations of coagulation markers and microvesicles than blood in the left ventricle.[64,65,76]

Left ventricular assist devices

There are no published data on PMV formation in implanted LV assist devices, although bleeding and thrombosis are a significant risk in this population.[77]

Hypothermia

Hypothermia below 31.3˚C during cardiac surgery is associated with decreased platelet function and decreased formation of PMV in heparinised blood, leading to increased bleeding.[78]

Patients undergoing CABG or trauma surgery with low pre-operative PMV counts are at an increased risk of blood transfusion.[39,79] Targeting PMV production and clearance to increase circulating numbers may reduce clinically important adverse effects such as bleeding, although larger studies with sampling of blood at multiple time points are required to better delineate the precise perioperative role of PMV. The infusion of PMV may be a potential future treatment for surgery-related bleeding with minimal transmission of pathogens or threat of immune response.[80]

Isolating and studying microvesicles

Many different methods are currently used to isolate microvesicles from whole blood. The International Society for Extracellular Vesicles is developing protocols for the extraction of

microvesicles to standardise research internationally.[6] The key process used in the majority of studies is the collection of whole blood, which is then centrifuged twice to obtain platelet-poor plasma (PPP). PPP can be stored at -80˚C and thawed prior to analysis by flow cytometry. Although many studies follow an interpretation of these methods, the differences in collection methods and centrifugation protocols have led to variation in reported microvesicle concentrations between studies.[51]

Blood for microvesicle studies should be taken with minimal shear stress; this can be achieved by a slow draw-back into a syringe from a central or arterial line or via venepuncture using a needle larger than 21 gauge.[8] This blood should preferably be collected into plastic tubes with sodium citrate anticoagulant prior to centrifugation, however other anticoagulants such as lithium heparin can be used. [8,81]

Centrifugation at low speeds may fail to obtain PPP, and overly high speeds can cause microvesicle loss or cause microvesicle doublets to form. Burnouf *et al* concluded that an initial centrifugation at 2,500g for 15 minutes within two hours of collection, followed by aspiration of PPP and subsequent centrifugation of the supernatant at the same speed and time results in low levels of platelets and less coupling of PMV.[82]

Conclusion

Pathologies such as Scott syndrome demonstrate, along with clinical and laboratory studies, that PMV have a crucial role in hemostasis. Although PMV could be used, among other predictors of transfusion risk such as pre-operative APTT and hemoglobin, to identify patients at risk of bleeding complications, current methods of identifying PMV populations are inadequate for the rapid and widespread use of PMV for prognostic purposes. However, the increased availability of flow cytometry equipment and refined protocols for isolating and investigating microvesicle numbers has enabled advances in our knowledge of sub-micron hematologic changes throughout cardiac surgery, though there is still much that is not known in this field. Future advances may see the widespread use of pre-operative PMV counts to

predict peri-operative bleeding complications, and as an assessment of platelet function generally, and transfusion of PMV as prophylaxis or treatment for hemorrhage.

Figure legends

Figure 1: The hematologic response to endothelial injury leading to the formation of a stable hemostatic plug.

Figure 2: In the non-activated state (left), the platelet membrane is held in asymmetry by movement of phosphatidylserine and phosphatidylethanolamine onto the inner surface. The increase in cytosolic Ca^{2+} associated with platelet activation results in the loss of asymmetry and the production of PMV.

Figure 3: PMV have an important role within the coagulation cascade. Circled factors bind with PS positive PMV to stimulate coagulation factor activation. Integrin and tissue factor are expressed on receptor-specific PMV membranes and these surface proteins interact with the cascade to enhance coagulation

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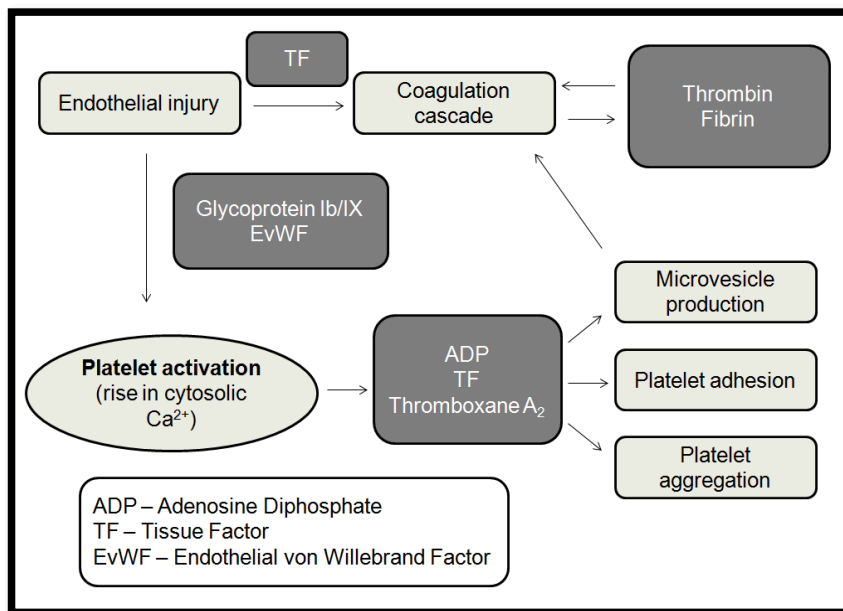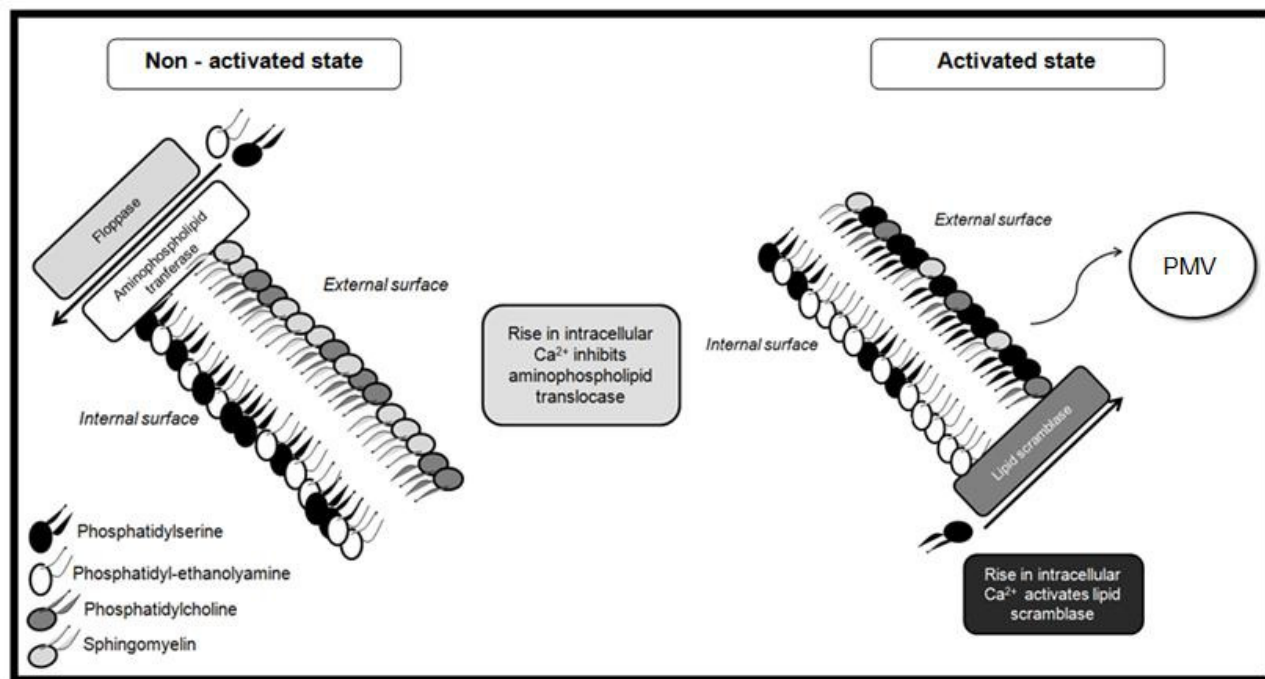
Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3