Chapter 4

Multidimensional Flow Cytometry for Testing Blood-Handling Medical Devices

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http://dx.doi.org/10.5772/intechopen.76437

Abstract

Blood handling devices such as left ventricular assist devices and total artificial hearts offer life-saving treatments for patients suffering from severe heart failure. Current devices have clinically proven that heart assist pumps are a safe and effective therapy, and indeed in many cases they are the only available method of treatment. However, current devices cause side effects including stroke, bleeding, infection, and thrombosis, preventing the technology from reaching its full potential. If the side effects could be reduced, then more patients could benefit from these devices. The complications are related to damage to blood cells and proteins as a result of contact with foreign materials and mechanical stress. There is a need for better devices with minimal blood impact to enable more patients to be safely treated; better tools, especially flow cytometry, could support the device development life cycle. In this chapter we review the clinical, in vivo, and in vitro flow cytometry data available for ventricular assist devices, conduct a gap analysis, and identify areas of future possibilities for device developers to establish new flow cytometry-based methodologies.

Keywords: multidimensional flow cytometry, translational research, ventricular assist, microparticles, multispecies

1. Introduction

Blood handling devices such as left ventricular assist devices and total artificial hearts offer life-saving treatments for patients suffering from severe heart failure. Current devices have clinically proven that heart assist pumps are a safe and effective therapy, and indeed in many

cases they are the only available method of treatment. However, current devices cause side effects including stroke, bleeding, infection, and thrombosis, preventing the technology from reaching its full potential [1]. If the side effects could be reduced, then more patients could benefit from these devices. The complications are related to damage to blood cells and circulating proteins as a result of contact with foreign materials and mechanical stress. There is a need for better devices with minimal negative effect on blood to enable more patients to be treated safely; better tools, especially flow cytometry, could support the device development life cycle.

The use of multidimensional flow cytometry during pre-clinical development of blood handling devices offers a powerful tool to monitor changes to erythrocytes, leukocytes, and platelets, as well as circulating mediators such as von Willebrand factor. A key challenge is the need to study these in cows, sheep and pigs which are used for pre-clinical studies. This is associated with markedly reduced reagent options compared to studies using human blood. While there are some species-specific antibodies suitable for flow cytometry, the preferential use of cross-reactive reagents and species non-specific tools enables multicolor panels to be developed that can be used with blood from multiple species. Such an approach also allows for comparisons at all stages of device development and implementation: in vitro, in vivo pre-clinical, and ex vivo clinical settings. Flow cytometry methods could also support personalised treatment strategies to potentially predict patients at risk of complications [2]. This could be prior to or following implantation of a device. Here, we will provide an overview of the development of flow cytometry tools to address this need including a review of work performed to date, as well as future possibilities for this technology platform.

2. Current use of flow cytometry for ventricular assist and total artificial heart research

The majority of the flow cytometry research performed in the area of blood-handling medical devices is to further the understanding of the complications that arise upon ventricular assist device implantation into heart failure patients. Since the aim of using flow cytometry during *in vitro* and

in vivo device testing is to maximise its haemocompatibility and minimise complications, it makes sense to start this review with the clinical data. The gaps in *in vitro* and *in vivo* research and development will then become evident and lay the foundation for the future possibilities for device developers.

2.1. Clinical flow cytometry data from ventricular assist devices

Flow cytometry has been used to analyse all major cellular components of the blood—i.e. erythrocytes, leukocytes, and thrombocytes—in VAD-patients.

2.1.1. Erythrocytes

Sansone et al. were the first group to use flow cytometry in the clinic to evidence VAD-related damage to erythrocytes, in addition to the standard method of measuring plasma free haemoglobin [3]. Patients implanted with continuous flow (CF) VADs (HVAD, HeartWare), showed significantly greater levels of $CD235^+$ erythrocyte MPs compared to controls (both age-matched healthy and patients with stable coronary artery disease, CAD). Their erythrocyte counts were not described, but the VAD-patients had significantly greater levels of free haemoglobin compared to controls. Increased levels of erythrocyte MPs have been found in patients suffering sickle cell disease and β-thalassemia major, which are diseases also characterised by haemolysis [3].

2.1.2. Leukocytes

From a flow cytometry perspective leukocytes have received more attention than erythrocytes. VAD-related leukocyte damage has been demonstrated using flow cytometry in all major leukocyte subsets. Using the pan-leukocyte marker CD45, leukocyte microparticles ($CD45^+$) were shown to be elevated in CF VAD patients compared to healthy and CAD controls [3]. This is indicative of overall leukocyte destruction and is supported by Woolley et al. who observed decreased total leukocyte counts in CF VAD patients [4]. In the same study, CD15+ neutrophils were found to become activated as measured by an increase in MAC-1 (CD11b) expression. The level of activated neutrophils was dependent on pump type: HeartMate II causing greater levels than HVAD and PVAD [4]. Neutrophil activation status might also influences the patient's susceptibility to infection as more HeartMate II patients than HVAD patients suffered from infection. The PVAD has a larger driveline exit area which contributes to infection rates, hence it cannot be directly compared to the other two pumps [5]. *In vitro,* neutrophils release $CD11b^+$ MPs during activation [6–8] and could therefore be the parental cell type for the $CD11b^+$ MPs that are elevated significantly in VAD-patients (mainly HeartMate II patients) compared to healthy controls [9].

Monocytes also become activated in VAD-patients with the expression of tissue factor (TF) increased significantly within the first month of pulsatile Novacor or HeartMate XVE support versus healthy controls [10, 11]. TF is a key element of the extrinsic coagulation cascade, and it is able to trigger coagulation, even with endothelial integrity virtually preserved. The major source of TF in blood is monocytes, and the expression is upregulated by for example lipopolysaccharides (LPS) [12]. As summarised by Angelillo-Scherrer: volunteers exposed to endotoxin, patients with meningococcal sepsis, and primates with Ebola fever, all show increased levels of $CD14^+/TF^+$ MPs, indicating a potential role for these MPs in disseminated intravascular coagulation associated with severe infections [12]. As driveline infections is a common problem in VAD-patients, there is a possibility that $CD14^+$ monocytes expressing TF and/or $CD14^+TF^+$ MPs, could be a thrombosis risk marker in patients with ongoing infection.

Lymphocytes are affected by both pulsatile and CF-VADs [13–15]. A general lymphopenia occurred in patients, implanted with the early pulsatile HeartMate XVE [14]. This was

accompanied by a significant reduction in the mean CD4/CD8 T cell ratios, and in the mean number of circulating CD4 T cells. The CD4/CD8 T cell ratio decreased rapidly within the first month and remained low at the 2 month follow-up assessment [14]. The decline in CD4 T cells was attributed to a heightened susceptibility to apoptosis as measured by surface expression of phosphatidylserine through annexin V binding [14]. These results were confirmed in a group of patients implanted with either the pulsatile HeartMate XVE or the Novacor, where the mean number of circulating CD4 T cells was significantly lower compared to medically managed heart failure patient controls. While the levels of CD8 T cells remained unaffected [15], both CD4 and CD8 T cells had increased CD95(Fas) expression and annexin V binding versus controls, indicating apoptosis. Furthermore, the LVAD-patients had a significantly greater risk of developing candidal infection compared to the controls or other patients undergoing cardiac surgery. Altogether, this suggests that the pulsatile LVADs cause T-cell defects, most notably CD4 T-cell defects, and that some of these defects are measurable through flow cytometry [14, 15]. How this translates to an effect on the function of T cells remains to be determined.

Patients with CF-VADs also have changes in their T cell levels. However, contrary to pulsatile LVAD-patients, those implanted with CF-VADs (specific device not published) and who suffered from infection had significantly higher levels of CD4+/CD25+ Tregs and increased lymphocyte reactive oxygen species (ROS) compared to VAD-patients without infection [13]. Whether these differences between pulsatile and CF VADs relates to pulsatility would be an interesting topic for further studies.

As far as we are aware, circulating B cells and NK cells have not been studied by flow cytometry in LVAD-patients. Nor have other minor populations such as dendritic cells or innate lymphoid cells. There are some data of the effect of VADs on B cells from in vitro studies (Schuster 2002) and these are discussed below.

2.1.3. Platelets

Platelets have been studied on their own and in microaggregates with leukocytes. Wilhelm et al. found that platelets were activated in patients with pulsatile VADs (Novacor and HeartMate XVE) compared to healthy controls [11]. This was measured as significantly increased CD62P expression. However, Dewald showed that increased platelet activation might not be due to the VAD as platelets in heart failure patients are already activated prior to implantation. This was shown using antibodies against CD62P, CD63, and antithrombospondin [16]. Similarly, Matsubayashi showed that CD62P and CD63 expression are elevated on platelets in Novacor-patients compared to healthy controls, but preoperative values were already high with no clear increase or decrease during implantation [17]. Further highlighting the impact of heart failure rather than VAD use on platelet activation *in vivo* granulocyte-platelet ($CD15^+/CD42b^+$) and monocyte-platelet ($CD14^+/CD42b^+$) aggregates were also increased significantly in the pulsatile VAD patients versus healthy controls before and after VAD implantation [11].

Platelet MPs also have been detected using flow cytometry in CF-LVAD patients [3, 9]. In 2010, Diehl P et al. showed that LVAD-patients, the majority of whom were implanted with the CF-LVAD HeartMate II, had significantly increased levels of CD31+/CD61+ platelet MPs compared to healthy controls [9]. Five years later, Sansone et al. showed that $CD31^+/CD41^+$ platelet MPs were elevated in patients 3 months post-implantation of a HVAD (a CF-LVAD) in comparison to both age-matched healthy controls and patients suffering from coronary artery disease (CAD) [3]. Hence, although the HeartMate II and the HVAD differ drastically in design, both were associated with platelet microparticle formation.

The focus has been on platelet activation and platelet MPs but these have so far not shown a utility as predictors of adverse events or stratifiers. However, there are other platelet parameters, all measurable by flow cytometry, that have potential as patient risk stratifiers, or even predictors, for bleeding complications in patients with CF-VADs [18–20]. These include significantly greater levels of reactive oxygen species, mitochondrial damage, surface phosphatidylserine (PS) expression/apoptosis, and significantly decreased expression of $\alpha 2b\beta 3$ on the platelet surface, in bleeders compared to non-bleeders [18–20]. The CF-VADs studied included the HeartMate II, Jarvik 2000, and the HVAD and no differences were found between the devices.

2.1.4. Endothelial cells and microparticles of unknown origin

$CD62E^+$ endothelial cell microparticles (EMPs) are increased in VAD-patients compared to healthy controls [3, 9] and CAD patients [3]. EMPs phenotyped as $CD31^+/CD41^-$, and $CD144^+$ are also elevated levels in VAD-patients [3].

Additional to the MPs of specific lineages described above—erythrocytes, leukocyte, platelet and endothelial—PS-expressing MPs of unknown lineage have been suggested as a potential biomarker of adverse events in VAD-patients implanted with a HeartMate II [2]. Patients who developed an adverse event, including ventricular tachycardia storm, non-ST elevation myocardial infarction, arterial thrombosis, gastrointestinal bleeding, and stroke had significantly higher levels of PS+ MPs than patients with no adverse events [2].

2.2. Pre-clinical in vivo haemocompatibility of blood-handling devices

The flow cytometry data from published pre-clinical *in vivo* studies of LVADs is focussed mainly on platelets with the exception of one study on leukocytes. There is no published data on erythrocytes.

2.2.1. Leukocytes

Snyder et al. have published the only *in vivo* leukocyte work wherein the aim was to develop assays for leukocyte-platelet aggregates and monocyte tissue factor expression [21]. Using an anti-bovine granulocyte antibody (CH138A) or anti-CD14 (TüK4) in combination with antibody CAPP2A (anti-ruminant CD41/61), calves implanted with CF-LVADs (HeartMate II

or EVAHEART) had significantly elevated levels of both neutrophil-platelet and monocyte-platelet aggregates compared to pre-operative [21]. Monocyte tissue factor expression monitored using an anti-bovine tissue factor antibody developed by Stephen Carson at University of Nebraska [22], also showed a dramatic increase immediately post-operatively and significantly elevated levels throughout the 30-day study [21].

2.2.2. Platelets

Bovine platelet activation and microaggregates have been studied using various markers in calves implanted with CF-VADs. Baker et al. developed a method using the antibodies BAQ56, BAQ125 and GC5A (platelet antigen, CD equivalent unknown, available from Washington State University) to measure platelet activation, and CAPP2A (anti-CD41/CD61) to measure platelet microaggregates in calves implanted with CF-LVADS (the Sun Medical centrifugal pump or the HeartMate II) [23]. Platelet microaggregates, i.e. platelet positive events (binding anti-bovine CD41/61 antibody) with forward scatter larger than single platelets, increased post-operatively and then showed some decline in calves implanted with Sun Medical whereas they remained elevated in the calves implanted with HeartMate II. Platelet activation increased post-operatively for both implants and remained elevated. BAQ56 provided the strongest signal and GC5 the weakest signal of the three antibodies, but BAQ125 and GC5 exhibited the strongest agreement with one another, and with the circulating microaggregates [23].

Snyder et al. used a modified version of Baker's methodology in two studies of calves implanted with the CF-LVADs—HeartMate II [24] and the EVAHEART [25]. Snyder showed that the microaggregate levels in surgical sham controls remained at pre-operative levels, confirming that the CF-LVAD and not the cardiac surgery cause elevated levels [24]. Microaggregate levels increased post-implantation, and decreased within the first week in all animals implanted with the EVAHEART [25], and in those animals implanted with the HeartMate II who had an uneventful post-operative course [24]. In calves who suffered adherent thrombi in the outflow region of the pumps, the microaggregate levels either remained elevated or increased before conclusion of the study [24].

Part of Snyder's modification was the inclusion of additional markers for platelet activation to address the limitations BAQ56, BAQ125, and GC5 with their unknown target antigens, namely Annexin V binding [24], anti-CD62P and anti-CD63 [25]. All platelet activation/apoptosis markers tested (BAQ125, GC5, Annexin V, CD62P, CD63) increased immediately after implantation and remained significantly elevated in CF-LVAD animals versus the pre-operative control [24, 25]. Those tested in sham animals (BAQ125, GC5, Annexin-V) decreased around 2 weeks post-operatively [24]. Although CD62P and CD63 successfully identified activated platelets in CF-LVAD calves, the signal was weaker than that of the BAQ125 and GC5 antibodies [25].

CAPP2A also binds to ovine platelets whereas anti-bovine platelet activation antibodies BAQ125 and GC5 do not. Hence, Johnson et al. used CAPP2A as a platelet lineage marker along with cross-reactive anti-human CD62P antibodies (clones Psel.KO.2.7 and Psel.KO.2.12)

to assess platelet activation in sheep implanted with paediatric CF-LVADs [26–28]. Johnson showed that, similarly to calves, platelet activation increases post-operatively in surgical sham control sheep and returns to pre-operative levels at around 2 weeks. The implanted sheep did not demonstrate a common pattern [28]. However, a finding also consistent with previous work was that platelet activation spiked in animals that suffered complications [26–28].

2.3. In vitro haemocompatibility of blood-handling devices

Similarly to the *in vivo* studies, the *in vitro* studies of VADs using flow cytometry are focussed on platelets, with some recent studies introducing leukocyte data. There is no published data on erythrocytes.

2.3.1. Leukocytes

Work from our group has shown that bovine leukocytes shed microparticles, measured as increasing levels of $CD45^+$ MPs (**Figure 1**), during *in vitro* pumping in the extracorporeal CF-LVADs CentriMag and RotaFlow, and the intracorporeal CF-LVAD VentrAssist [29, 30]. We have also shown that $CD45^+$ MPs increase significantly when sheep rather than bovine blood was pumped with the CentriMag [31]. Subtypes of leukocyte MPs were discovered

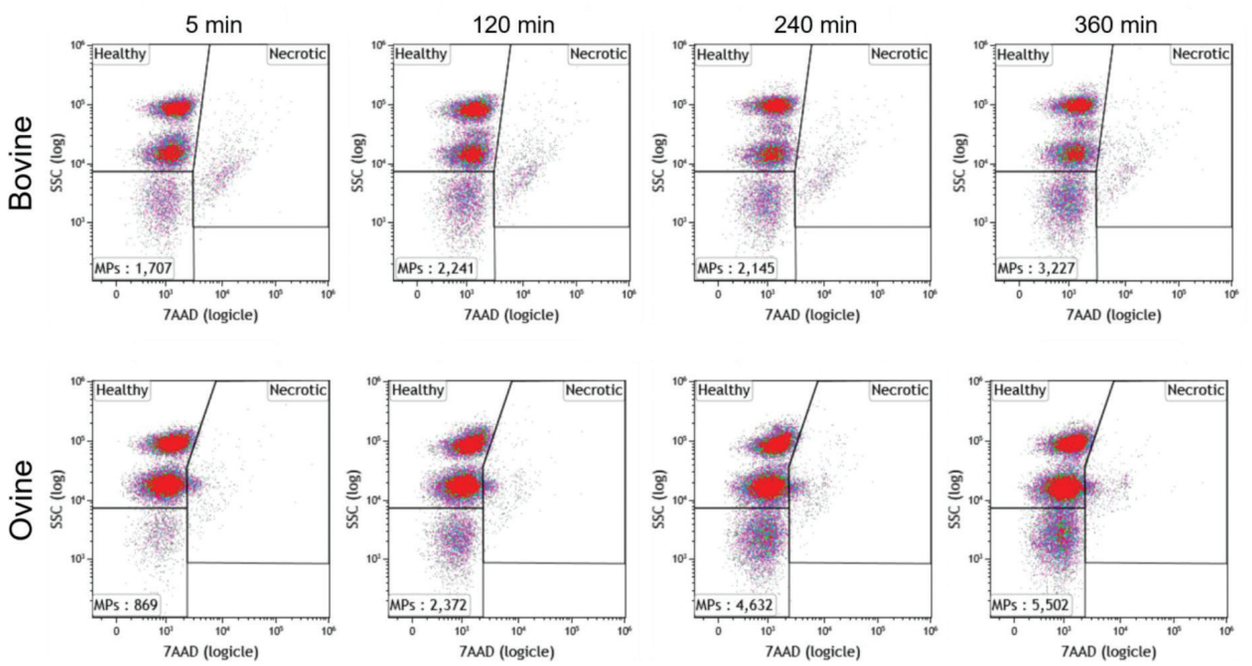

Figure 1. Flow plots showing bovine and ovine blood pumped through the CentriMag. Whole blood was collected into CPDA-1 anticoagulant primed with antibiotics/antimycotics and gentamicin. Blood was diluted with PBS to achieve a haematocrit of 30±2% according to ASTM standards and entered into the mock circulatory loop. The CentriMag was operated at a speed of 2200 rpm, flow of 5 L/min, and pressure 100 mmHg for both species. Samples were removed every 2 h and stained with CD45-PE and 7AAD. CD45+ events were gated on a SSC vs. 7AAD plot and events with a low SSC were identified as leukocyte-derived microparticles (MPs).

in sheep blood during *in vitro* VAD testing using antibodies cross-reactive with human and bovine blood. The main subtypes were CD11b^{bright}/HLA-DR^{-} and CD11b^{dim}/HLA-DR^{+}, discovered using a four-colour panel (**Figure 2**), and we suggested that these are derived from granulocytes and lymphocytes, respectively [31].

2.3.2. Platelets

The first flow cytometry assessments of platelet activation and microaggregates during *in vitro* testing in CF-LVADs was carried out by Johnson C et al. using sheep blood. Activation was assessed using CAPP2A as a lineage marker and anti-CD62P (clone Psel.KO.2.7), and was found to increase throughout the duration of the test of the PediaFlow [28]. We have used CAPP2A, BAQ125, and Annexin V to assess platelet activation in bovine blood in the CentriMag, but did not find any significant activation. However, the CentriMag has a magnetically levitated impeller, resulting in minimal heat, and also large gaps minimising blood damage, so these findings were not surprising [30] (**Figure 3**).

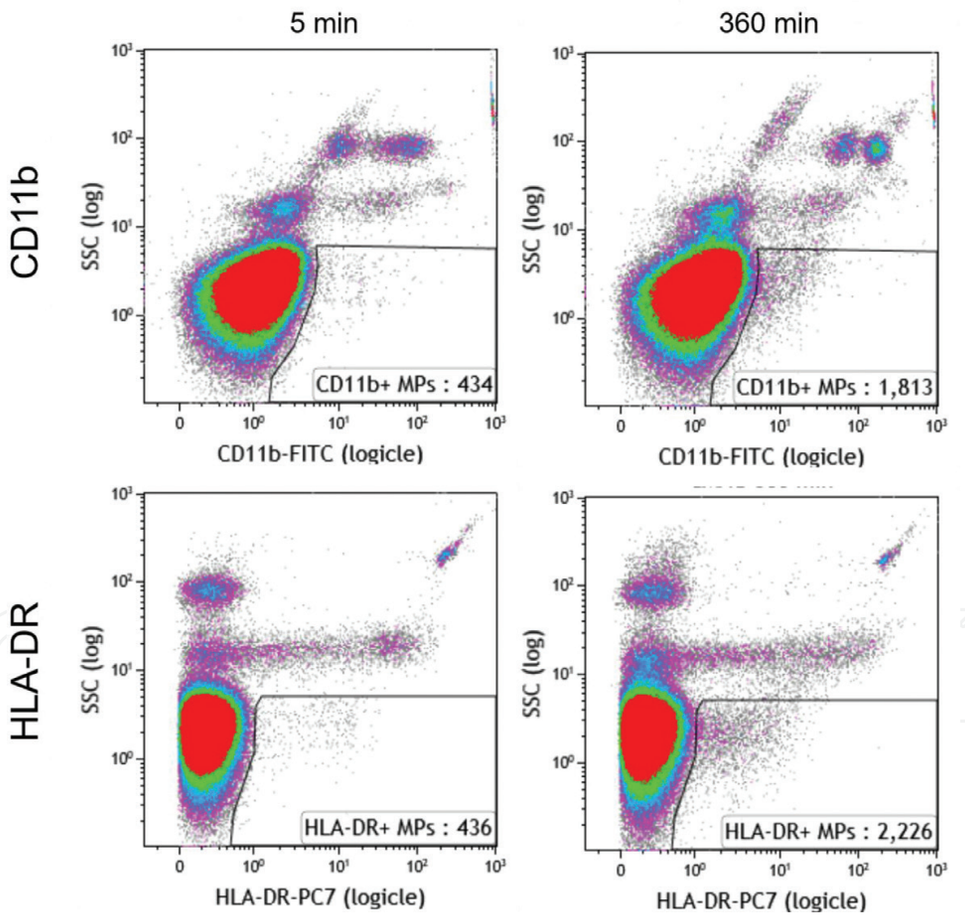

Figure 2. Flow plots showing ovine blood pumped through the CentriMag operated at a speed of 2200 rpm, flow of 5 L/min, and pressure 100 mmHg. Whole blood was collected into CPDA-1 anticoagulant primed with antibiotics/antimycotics and gentamicin. Blood was diluted with PBS to achieve a haematocrit of 30±2% according to ASTM standards and entered into the mock circulatory loop. Samples were stained with CD11b-FITC and HLA-DR-PC7. Events with a low SSC and positive for these markers were gated as CD11b or HLA-DR positive microparticles (MPs).

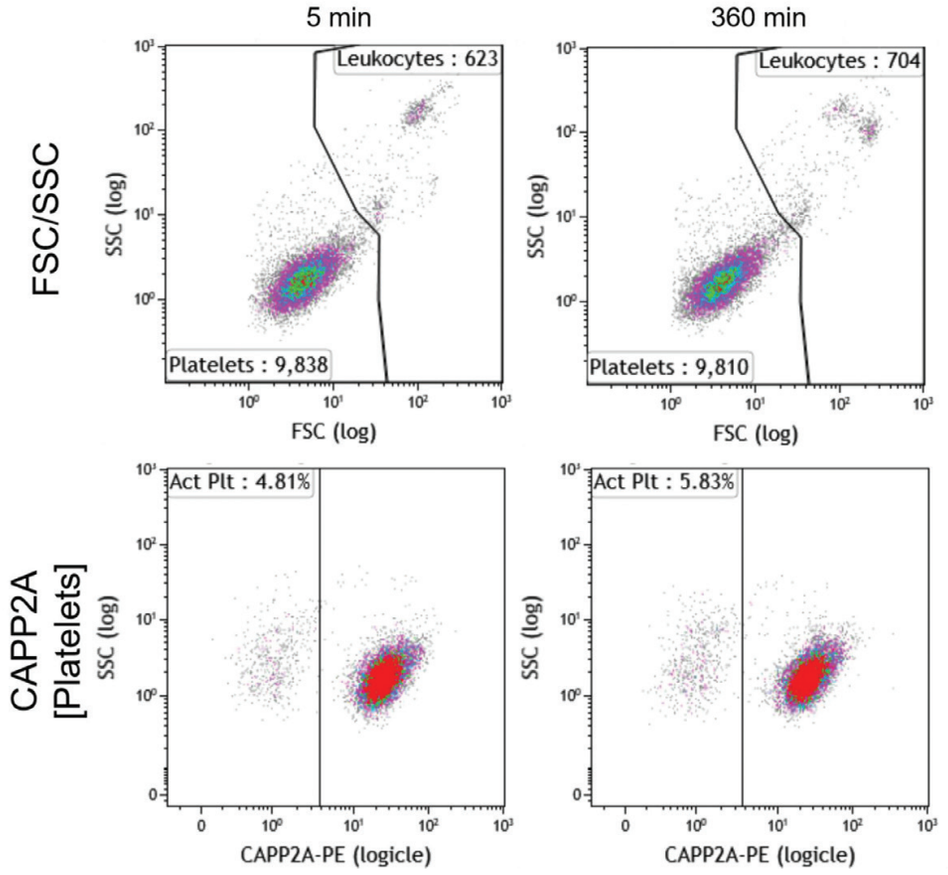

Figure 3. Flow plots showing bovine blood pumped through the CentriMag operated at a speed of 2200 rpm, flow of 5 L/min, and pressure 100 mmHg. Whole blood was collected into CPDA-1 anticoagulant primed with antibiotics/antimycotics and gentamicin. Blood was diluted with PBS to achieve a haematocrit of 30±2% according to ASTM standards and entered into the mock circulatory loop. Samples were stained with CAPP2A-PE, a marker for resting platelets. Forward and side scatter plots were used to identify platelets. CAPP2A negative platelet events were identified as activated platelets (Act Plt).

3. Future possibilities for device developers

Although flow cytometry has been used clinically to study phenotype, activation status and MPs of the main circulating cell types in patients implanted with mechanical circulatory support devices, the use of flow cytometry for pre-clinical *in vivo* and *in vitro* studies has been very limited. As can be seen in **Table 1**, there are many potential gaps that could be filled by developing assays for *in vitro* use, as well as *in vitro* assays to translate to the pre-clinical and clinical *in vivo* setting. The *in vitro* setting will always provide the worst case scenario, as the pumped blood volume is 10 times less than in the VAD-patient and the blood components are therefore experiencing an increased amount of pumping, not to mention experiencing plastic tubing instead of endothelial coated vasculature. In addition, there is no supply of oxygen or nutrients other than what is already present in the plasma, and there is no efficient removal of waste products or damage/dead cells. Cells are therefore more vulnerable *in vitro*. Therefore, if a VAD induces detectable cellular damage clinically, it will most certainly be measurable in

Cell type	Assay	In vitro		In vivo		Clinical
		Ovine	Bovine	Ovine	Bovine	
Erythrocytes	**CD235+ MPs**					✓
Leukocytes	**CD45+ MPs**	✓	✓			✓
Granulocytes	**CD11b + MPs**	✓				✓
	CD11b + expression					✓
Monocytes	**TF expression**				✓	✓
Lymphocytes	**Subset levels: CD4+, CD8+, CD4+/CD25+**					✓
	ROS, apoptosis					✓
	HLA-DR+ MPs	✓				
Platelets	**Activation**	✓	✓	✓	✓	✓
	Phosphatidylserine		✓		✓	✓
	MPs					✓
	ROS, etc.					✓
	Microaggregates			✓	✓	
GRA-PLT	**Microaggregates**				✓	✓
MON-PLT	**Microaggregates**				✓	✓
Unknown	**Phosphatidylserine MPs**					✓

Table 1. Gap analysis of assays for evaluating blood-handling devices.

an *in vitro* test. Platelet activation is so far the only assay that has been fully reverse-translated from bedside to bench, but there is no reason to believe that it should not be possible to create suitable flow cytometry assays for erythrocytes and leukocytes for all stages in the product development process. The benefits of implementing such assays for the devices in development could be identification of designs with minimal impact on blood components, and thus a reduction of the blood-damage related life-threatening complications so commonly seen in VAD-patients including bleeding, stroke, device thrombosis and infection.

3.1. Erythrocytes

The VADs in current use are designed to minimise high shears to blood for prolonged durations to avoid haemolysis. However, erythrocytes demonstrate impaired capacity to deform when exposed to shear stress well below the "haemolytic threshold" [32]. Thus, there is a need for assays that can pick up more subtle damage to erythrocytes. We propose that quantifying RBC MPs as a possible means of assessing sub-haemolytic damage should be investigated to see whether or not it can fill this need.

3.2. Leukocytes

Activated $CD15^+CD11b^+$ neutrophils, have been detected in VAD-patients and clinical data shows that the activation status might influence the patient's susceptibility to infection [5]. With additional evidence that $CD11b^+$ MPs are increased in VAD-patients, and generated during *in vitro* pumping in ovine blood, it makes sense to propose further investigations into its utility as a stratifier to identify those at the highest risk of developing infections in order to offer prophylaxis, thereby reducing infection rates.

VAD-related increases in monocytes expressing TF have been demonstrated both clinically [10, 11] and *in vivo* [21]. $CD14^+/TF^+$ MPs are elevated in primates, healthy volunteers, and patients subjected to infectious stimuli that could result in disseminated intravascular coagulation [33]. Thus, we propose that in VAD-patients with ongoing driveline infections, the presence of $CD14^+/TF^+$ monocytes/MPs potentially could identify those patients at risk for a thrombotic event in order to offer prophylaxis. Assays could also be developed for *in vitro* testing of devices to see if the combination of design, foreign material and/or shear stress in different device models results in TF-expression. However, care should be taken when assessing $CD14^+TF^+$ MPs as false positive results can be generated by the antibody preparations used [34].

The lack of pulsatility has been suggested as a factor that could be contributing to the complications related to aortic valve insufficiency, gastrointestinal bleeding, stroke, pump thrombosis, and haemolysis [35]. A link between pulsatility and blood damage has so far only been described for the blood coagulation protein von Willebrand Factor (vWF), which appears to degrade more in patients with pulsatile compared to non-pulsatile VADs [36]. As our group has shown, vWF degradation caused by shear stress in vitro can be assessed using flow cytometry a flow cytometry-based ristocetin assay [37]. However, it appears as if T cells could be another missing link needed to describe the effects of non-pulsatile [13] and pulsatile [14, 15] flow on the blood. Therefore, we propose that assays for T-cells are established for sheep and cow blood in order to study the effects of pulsatility, in order to gather more scientific evidence that could be used to interpret the clinical data.

Other leukocyte subsets of interest to develop methods for studying would be B-cells. Schuster et al. described that the T-cell apoptosis observed in patients with pulsatile VADs, was induced by a B-cell response to polyurethane, a material commonly used for the membranes in pulsatile devices [38, 39]. Continuous flow devices are typically made from metals and ceramics, and do not contain polyurethane. Hence, pulsatility studies should be made in combination with various biomaterial controls, including for example titanium alloys commonly used in VADs [40, 41].

3.3. Platelets

In vitro and *in vivo* platelet studies have so far been limited to studying activation by increased expression of platelet markers, but the expression of platelet activation surface markers has so

far failed to be linked to clinically relevant events. However, Mondal's portfolio of potential risk stratifying platelet parameters [18–20] are of importance to study *in vitro*. Hence, we propose that effort be made to translate Mondal's methods to bovine and/or sheep blood for *in vitro* studies of platelet reactive oxygen species, mitochondrial damage, and α2bβ3 expression to investigate any potential links between device design and its influence on platelet health. Since platelets are numerous in blood, very small volumes can be used to analyse them. In combination with a multidimensional approach to assess several parameters simultaneously, it would be a very attractive addition to the care pathway for patients with heart failure pre- and post-VAD implantation.

3.4. Cytokines

Cytokines have not been assessed using flow cytometry in a VAD-setting, but recent clinical data on TNFα-levels in VAD-patients might spur a change. High levels of TNF-α were associated with increased risk of bleeding complications in patients with CF-VADs (HeartMate II or HVAD) [42]. Measurement of TNF-α by flow cytometry in whole blood would offer a much more rapid method, a lower sample volume required, and could be combined with other cellular markers of interest into a multidimensional approach [43].

3.4.1. Overcoming practical hurdles

A reason for hesitating in this approach might be the limited availability of reagents for animal blood. However, progress has been made recently in this field by exploiting the cross-reactivity of some antibody clones. We identified the utility of an antibody clone targeting CD62P, clone Psel.KO.2.5, that could measure shear-induced platelet activation in human, bovine, ovine, and porcine blood [44]. Johnson et al. also made significant contributions in identifying cross-reactive platelet antibodies against bovine and ovine platelets [45]. We have used antibodies cross-reactive against bovine and human targets in developing a multicolour flow cytometry panel for sheep leukocyte MPs released during *in vitro* VAD-pumping. This panel should offer translation to both the pre-clinical *in vivo* and clinical setting [31]. To address the cost of antibodies and reduce the need for additional haematology analysers, we have also demonstrated that flow cytometry in combination with DNA dyes and counting beads could be used to assess complete leukocyte counts and viability in bovine, ovine, and human blood [46]. Although personal flow cytometers have been introduced lately, they are still not widespread and many investigators rely on the use of core facilities for flow cytometry acquisition and analysis. Where the animal lab is not in close proximity to the flow cytometry core facility there may be a need to preserve the animal blood prior to shipping for analysis. CytoChex and Streck stabilising fluid were developed to address the need to preserve CD4 antigens for HIV drug trials in remote locations for central analysis during clinical trials, but have now been shown to stabilise several human leukocyte antigens [47–49]. We are developing protocols for the use of these reagents with bovine and ovine blood to stabilise samples for several days and enable subsequent flow cytometry analysis of platelet and leukocyte parameters (in submission).

http://dx.doi.org/10.5772/intechopen.76437

4. Conclusion

Assessment of red blood cell damage through measurement of plasma free haemoglobin is a key assay required by regulatory bodies in the development of new blood-handling devices. However, more sophisticated flow cytometry screening methods that enable evaluation of a wider array of blood cell types and blood borne proteins could be used in combination with other design tools to inform iterative design improvements. Such an approach would enable device developers to progress new designs that have minimal total blood trauma, and ultimately put safer devices on the market. The use of multiparametric flow cytometry in the design cycle and clinical use of medical devices that contact blood is in its infancy. Clearly, there are many advantages to such an approach. The key advantage is the ability to analyse the phenotype of multiple haematopoietic cell populations simultaneously in small volumes of blood. Such methodology would enable the same analysis protocols to be used at all stages of design and implementation: in vitro studies, pre-clinical studies in different animal species, and then in clinical studies once devices are implanted in humans. Multiparametric flow cytometry also enables the incorporation of functional outputs such as cell viability, apoptosis, reactive oxygen species (ROS) production and cytokine production. These are critical if the impact of device-associated biomaterials and shear stress on haematopoietic cells is to be determined; there might not be phenotypic changes but cell function might be affected dramatically. The main challenges to implementing multiparametric flow cytometry in this setting relate to the availability of reagents that are truly multi-species and enable a single methodological approach to be used across the device development life cycle. This is more challenging when it comes to cell phenotyping that typically requires antibodies to specific cell surface markers but easier to implement for functional assays related to cell viability/apoptosis and measures such as ROS. Other challenges relate to flow cytometer availability to the research and development team as that will dictate fluorochrome choice and assay design. Also, data analysis becomes demanding as the number of parameters studied simultaneously increases and machine learning approaches need to be developed alongside development of multiparameter strategies. Rapid advances in fluorochrome technology and the development of mass cytometry facilitate a multiparametric approach but there remain limitations in fluorochrome choices for reagents targeting surface markers and cytokines of cells from animal species such as cows, pigs and sheep. Demand for these reagents will drive their availability but this demand will only occur if the device development community adopt standardised approaches. Underpinning this would be a requirement from regulatory bodies for flow cytometry-based analysis of cell health and function as part of the development and evaluation of blood handling medical devices.

Conflict of interest

Ina Laura Pieper is a past employee of Calon Cardio-Technology Ltd., and a current employee and shareholder of Scandinavian Real Heart AB. Gemma Radley is an employee of Calon Cardio-Technology Ltd. Catherine A Thornton has no conflicts of interest.

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