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IN VITRO BENCHMARKING STUDY OF VENTRICULAR ASSIST DEVICES IN CURRENT CLINICAL USE

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Abstract:

Background:

Left ventricular assist devices (LVADs) offer live-saving therapy to transplant-ineligible heart failure patients. A major limitation of the technology includes pump thrombosis, bleeding, and recurrent infection that prove difficult to predict from *in vivo* animal testing. Shear stress introduced by the LVAD affects more than just haemolysis since platelets, leukocytes, and plasma proteins all contribute to the propensity for complications. It is important to assess overall damage by a new device against a base line as early as possible in the development process so that design iterations can be made if required.

Methods:

Explanted VADs currently in clinical use (HeartMate 2 and HVAD) were carefully cleaned, inspected, and run at 5 L/min and pressure at 100 mmHg in a standard 500 mL mock circulatory loop using bovine blood. The CentriMag was used as a control pump due to its low blood damage profile. Samples were collected at regular intervals and the following analysed: complete cell counts; haemolysis; platelet activation; leukocyte-derived microparticles (LMPs); and von Willebrand factor (vWF) degradation.

Results:

The HeartMate 2 had the highest levels of haemolysis and platelet activation after 6 hours compared to the HVAD and CentriMag. A decreased granulocyte count, high numbers of LMPs and $CD11b^{Bright}HLADR^{-}$ LMPs, and decreased vWF collagen binding activity was most evident in the HVAD.

Conclusions:

The results indicate that it is possible to observe differences between different pump designs during *in vitro* testing that might translate to clinical performance. This study demonstrates the importance of developing standard *in vitro* total blood damage methods against which device developers could use to modify design to reduce complication risk long before implantation.

Introduction

Heart failure (HF) currently affects at least 26 million people worldwide (1) with a 25% expected increase in prevalence by 2030 (2). Healthcare costs for chronic heart failure (CHF) accounts for around 2% of the UK NHS expenditure (3) and $30 billion per year in the USA (4). The cure for HF is a heart transplant which has excellent 1-year (93%) and 5-year survival (88%), and functional capacity (5). However, with only approximately 7000 global transplants performed annually (6) and a vast majority of patients classed as ineligible for transplant due to age, weight, and comorbidities (7), there is great need for an effective long-term alternative therapy.

Implantable left ventricular assist devices (LVADs) have emerged as mainstream treatment for severely symptomatic heart failure in selected non-transplant eligible patients (8, 9). Survival data from INTERMACS suggest LVAD outcomes now rival transplantation particularly in patients with ischaemic cardiomyopathy (10). Pump thrombosis was once a persistent, major limitation of this technology (11) however newer designs have managed to eradicate this (12). Despite improvement in thrombosis, other complications such as GI bleeding, and LVAD-related infection still remain problematic (13) and might be difficult to predict from *in vivo* testing in sheep or calves. Nonetheless, extensive pre-clinical testing is mandatory prior to clinical application of any new LVAD (12) with as much as possible learnt from *in vitro* work before any animal studies. Haemocompatibility testing plays a major role in this but the shear stress encountered by blood in transit through a rotary pump can affect other cell types and large proteins in the plasma (14). Therefore, blood testing requires more than evaluation of haemolysis since platelets, leukocytes and plasma proteins all contribute to the propensity for infection, gastrointestinal bleeding and thrombus formation (15-18). It is important to assess overall damage by a new device against a base line as early as possible in the development process so that design iterations can be made if required.

Current temporary extracorporeal blood pumps, such as the widely used CentriMag® centrifugal pump (CP), may cause less blood damage than implanted LVADs because of the lack of size constraints. Extracorporeal use allows for larger slower moving rotors which provide lower shear stress. Combined with relatively low cost replaceable pump heads and convenient flow connectors this makes

extracorporeal blood pumps a good choice as a baseline control for incorporation into a disposable test loop for *in vitro* haemocompatibility testing.

Previously, we have created a blood damage profile of the CentriMag® using assays including haemolysis, haematology (blood cell counts), white blood cell microparticles and von Willebrand factor (vWF) degradation (19). This showed very low haemolysis levels (NIH of 0.0011 g/100L), no changes in blood cell counts, but an increase in white blood cell microparticles and degradation of vWF. As the CentriMag pump has widespread clinical use, it is a good baseline control to compare the implantable LVADs against. Blood damage on par with the CentriMag can be considered clinically acceptable whereas significant increases in damage could go some way to explain clinical complications for specific pumps designs.

The purpose of this study was to compare the blood damage profiles of the CentriMag, the HeartMate 2 (HMII) and the HVAD, using the assays developed in house for haemolysis, haematology, white blood cell microparticles, platelet activation and von Willebrand factor structure and function (19-21).

Methods

Preparation of test blood

Blood was collected and prepared as described previously (19). Bovine peripheral blood was collected from the carotid artery during slaughter by gravity-filling a bottle primed with 14% citrate phosphate dextrose adenine (CPDA-1) anticoagulant and 50 mg/L gentamycin and 10 mL/L antimycotic solution (Sigma-Aldrich, Poole, UK). The blood was diluted with phosphate buffered saline (PBS, Life Technologies, Paisley, UK) as required to reach a haematocrit of 30 ± 2% to standardise samples (22). The blood was used in the experiments within 4 hours of collection.

Device operation and specifications

The following VADs were included in the study: 3 x HVAD (Medtronic, Framingham, MA, US), 3 x HMII and CentriMag CP (Abbott, Thoratec, Pleasanton, CA, US). The CentriMag CP used throughout this test was used directly out of packaging and used exclusively for *in vitro* testing with bovine blood. Explanted HMII and HVAD were subjected to rigorous cleaning to ensure no biological matter remained that could affect the test. The cleaning process involved sonication of the pumps in 5% Neutracon solution (Decon Laboratories, Location) at 50°C for 10 min, followed by a dH_2O rinse then submerged briefly in 100% ethanol to remove water and allowed to dry. All pumps were tested using an *in vitro* test circuit under constant haemodynamic conditions in accordance with ASTM Standards as described previously by our group (19).

Haemolysis assay

Haemolysis was measured using the Harboe assay as described previously (20). The CentriMag has a very low and reproducible level of haemolysis (19). Static blood contained in a bag in a +37°C water bath was included as a negative control in each experiment.

Automated haematology analysis

Complete blood counts were measured using the veterinary analyser Abacus Jr Vet 5 (Diatron, Budapest, Hungary) at time points 5, 120, 240, and 360 min in triplicate.

Platelet activation

A positive control for platelet activation was created by treating a baseline blood sample with 4 µM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 60 min at room temperature with gentle agitation. To fix the activated platelets, 100 µL of PMA-treated blood was added to 100 µL Streck Cell Preservative (Streck Laboratories, City, CA, US), inverted 10 times and stored over night at +4°C. Baseline blood along with blood samples collected at time points 5, 120, 240, and 360 min were fixed in the same way. 20 µL of fixed blood was stained with CAPP2a (1.0 mg/dL, an anti-ruminant CD41/61 antibody (23)) and analysed using flow cytometry as described previously (19). CAPP2a is expressed on resting inactivated platelets and decreased with platelet activation. Activated platelets were noted as CAPP2a-negative ($CAPP2a^-$) events.

Leukocyte microparticles

Blood samples from time points 5, 120, 240, and 360 min were analysed for $CD45^+$ microparticles as described previously by our group (24). To characterise the leukocyte microparticles, samples were also stained with a panel including lineage and activation markers. The antibodies used were anti-CD11b-FITC (AbD Serotec, Oxford, UK), anti-CD21-PE (AbD Serotec), anti-CD14-BDV500 (BD Bioscience, Oxford, UK), anti-HLADR-PE-Cy7 (eBioscience, Hatfield, UK), MM1A for bovine T cells and MM20A for bovine granulocytes (both from WSUMAC, Pullman, WA, USA). These antibodies were conjugated in the lab using Lightning Link APC kit for MM1A (Novus Biologicals, Abingdon, UK) and the Zenon Pacific Blue Conjugation Kit for MM20A (Life Technologies) according to the manufacturers' instructions.

All samples were acquired with a Navios flow cytometer equipped with three lasers (violet: 405 nm, blue: 488 nm, red: 638 nm), the standard filter configuration, and with *Navios Cytometry List Mode Data Acquisition and Data Analysis Software Navios Cytometer 1.2* software (Beckman Coulter, High Wycombe, UK). Forward scatter (FSC), side scatter (SSC), and fluorescent voltages were set using unstained samples and all samples were recorded for 60 sec. Stained AbC bead samples were acquired using a gate around the beads and capturing 10,000 events. Analysis was performed in Kaluza 1.5a (Beckman Coulter) as described previously (24).

The instrument was maintained using daily cleaning procedures recommended by the manufacturer throughout the study period. The quality control used the Flow-Check and Flow-Set Pro Fluorospheres (Beckman Coulter) and the protocols used were set-up by the manufacturer's technical support engineers.

vWF collagen binding activity

The vWF collagen binding activity (vWF:CBA) in the samples was assessed using a Zymutest vWF:CBA enzyme-linked immunosorbent assay (ELISA). Absorbance was measured at 450 nm (POLARstar Omega) and standardised to the static control.

vWF immunoblotting

To visualise vWF multimers, platelet-poor plasma (PPP) was prepared by centrifuging 1 mL aliquots of whole blood at 4700 x g for 7 min. The supernatant (PPP) was removed into a separate tube for analysis. Samples were fractionated under non-reducing conditions using sodium dodecyl sulphate (SDS)-agarose gel electrophoresis, transferred onto polyvinylidene difluoride (PVDF) membrane and probed using anti-vWF antibody.

Statistical methods and analysis

Averages and standard deviations were calculated for all parameters and time points. For haemolysis and platelet activation, the background levels observed at 5 minutes were subtracted from all other measurements. Leukocyte microparticles and complete blood counts were divided by the static control at time point 5 minutes to evaluate the relative increase or decrease caused by duration of pumping whilst minimising donor-to-donor variability. For haematology, vWF immunoblotting and vWF:CBA, the data were expressed as a percentage of the static 5 minute control The dataset consisted of repeated measurements of blood samples across three pumping conditions and 4-7 time points (7 for haemolysis, 4 for the other assays).

Since the differences between the different pumps were so stark and changes monotonic and cumulative we felt that complex models were unnecessary. Since damage is cumulative we performed a simple comparison of the pumps at the 360 minute mark, using a one-way ANOVA. Post hoc tests were

performed using least significant difference (LSD), which is both simple to interpret and appropriate for such a small number of groups. Difference between pumps were reported as effect size (η^2) and as a 95% confidence interval. Temporal trends were tested using Linear Regression. A 5% level of significance was used throughout. All statistical data analysis was performed in IBM SPSS version 25 (SPSS, Inc., Chicago, Illinois, US).

Exclusion criteria

Any tests during which the flow rate failed to meet the target range (4.75 – 5.25 L/min) were excluded from further analysis. The CentriMag pump was used in each test as a control of the experimental conditions. If the normalised index of haemolysis (NIH) value for the CentriMag after completion of the test was ≥0.002 g/ 100 L, the blood was considered of low quality and the experiment (including all data from the haematology analyser and flow cytometry assays) was excluded from the analysis. Tests where the platelets did not activate in response to chemical stimulation with PMA were excluded.

Results

Haemolysis

NIH in both the HMII (n = 12) and the HVAD (n = 11) at 360 min was significantly higher than in the CentriMag (n = 25) ($p < 0.0001$; Figure 1A). The NIH is calculated from the pfHb and these followed the same trend: HMII ($\eta^2 = 0.84$; 95% CI: 107.82 – 137.25) and HVAD ($\eta^2 = 0.84$; 95% CI: 34.71 – 62.88) significantly higher than the CentriMag ($p < 0.0001$) at 360 min (Figure 1B). Also, HMII and HVAD pfHb was significantly higher than that of the CentriMag after only 60 min (HMII: $\eta^2 = 0.63$; 95% CI: 12.35 – 18.87, $p < 0.0001$ and HVAD: $\eta^2 = 0.63$; 95% CI: 0.55 – 6.79, $p = 0.022$).

Haematology

All baseline values for red blood cells, platelets, white blood cells and their subsets were reviewed against normal reference ranges (25). Haematology assessment was done at all time points for the CentriMag (n = 25), HMII (n = 12), and HVAD (n = 11). Red blood cell counts were unaffected by all pumps (Figure 2A). Total leukocyte counts decreased significantly in all pumps over time (linear regression $p < 0.001$, Figure 2B). Platelet counts were significantly greater in the HMII ($\eta^2 = 0.35$; 95%

CI: 0.08 - 0.40, p = 0.004) and HVAD (η^2 = 0.35; 95% CI: 0.12 - 0.36, p < 0.0001) and compared to the static control (Figure 2C). The analysis of the white blood cell subsets revealed that a decline in neutrophils is the main contributor to this downward trend. The HVAD granulocyte count was significantly lower compared to the static control (η^2 = 0.28; 95% CI -0.2442 – -0.9430, p = 0.001) and significantly lower compared to the CentriMag in both HMII (η^2 = 0.28; 95% CI: -0.0437 – -0.9651, p = 0.011) and HVAD (η^2 = 0.28; 95% CI: -0.3158 – -1.0145, p = 0.001; at 360 min (Figure 2D). Monocyte and lymphocyte counts were unaffected by the pumps (Figures 2E & F). This phenomenon of decreased leukocyte counts and increased (false positive) platelet counts is due to the release of microparticles from leukocytes, as demonstrated previously (21). To conclude, the HVAD performed poorest when it comes to white blood cell handling, especially the handling of granulocytes.

Platelet Activation

Platelet activation was measured at all time points for the CentriMag (n = 17), HMII (n = 8), and HVAD (n = 10). Platelet activation was elevated significantly in the HVAD at 240 min (η^2 = 0.21; 95% CI: 0.04 – 0.44, p = 0.017) and 360 min (η^2 = 0.30; 95% CI: 0.02 – 0.61, p = 0.035) and the HMII at 240 min (η^2 = 0.21; 95% CI: 0.0826 - 0.5024, p = 0.007) and 360 min (η^2 = 0.30; 95% CI: 0.32 – 0.95 , p < 0.0001) when compared to the static control (Figure 3).

Leukocyte-derived Microparticles

Leukocyte-derived microparticles were assessed at all time points in CentriMag (n = 15), HMII (n = 10), and HVAD (n = 9). The levels of microparticles were assessed in each pump and divided by those found in the static control at 5 min. Both HMII (η^2 = 0.79; 95% CI: 18.51 – 33.09, p < 0.0001) and HVAD (η^2 = 0.79; 95% CI: 30.27 – 44.32, p = 0.004) had elevated levels of LMPs in comparison to the static control after 5 mins. When compared to the CentriMag, the HMII and HVAD showed significantly increased levels of LMPs at all time-points (p < 0.0001; Figure 4).

Activated Leukocyte Microparticles

Our group has previously characterised the activation status of LMPs in ovine blood pumped using the CentriMag and identified three distinct populations: $CD11b^{Bright}HLADR^{-}$; $CD11b^{Bright}HLADR^{+}$; and $CD11b^{Dull}HLADR^{+}$ (24). Here, we have examined this using bovine blood with activated leukocyte-

derived microparticles assessed at all time points in CentriMag (n = 13), HMII (n = 9), and HVAD (n = 8).

The $CD11b^{Bright}HLADR^{-}$ LMPs were increased significantly after 240 min in the HMII and HVAD ($p < 0.0001$ for both; Figure 5A) compared to the CentriMag and static control. The $CD11b^{Bright}HLADR^{+}$ LMPs were increased significantly after 120 min in the HMII ($\eta^2 = 0.61$; 95% CI: 3.98 – 16.45, $p = 0.003$) and HVAD ($\eta^2 = 0.61$; 95% CI: 15.77 – 30.52, $p < 0.0001$) (Figure 5B) compared to static control. The pattern of activated MPs was changed with the $CD11b^{Dull}HLADR^{+}$. After 120 min, all pumps had a significantly increased $CD11b^{Dull}HLADR^{+}$ LMP population (CentriMag, HVAD & HMII: $p < 0.0001$; Figure 5C) compared to the static control.

To determine whether these activated microparticles were granulocyte-derived, they were analysed for MM20A expression (Supplementary Figure 1). Expression of MM20A on the $CD11b^{Bright}HLADR^{-}$ population that increased significantly to 360 min in all pumps (Supplementary Figure 1A; CentriMag, HMII, & HVAD: $p < 0.0001$) in keeping with the results in Figure 5A indicate that this microparticle subset is granulocyte-derived. This was not the case for $CD11b^{Bright}HLADR^{+}$ and $CD11b^{Dull}HLADR^{+}$ microparticles (Supplementary Figure 1B&C).

vWF CBA

The function of von Willebrand factor, as measured by its collagen binding activity, steadily decreased with increased pumping time in all pumps ((CentriMag (n = 8), HMII; (n = 6), and HVAD (n = 8)). vWF:CBA significantly decreased in the HMII ($\eta^2 = 0.451$; 95% CI: -0.02438 – -0.17998, $p = 0.013$) and HVAD ($\eta^2 = 0.45$; 95% CI: -0.06– -0.20, $p = 0.001$) after only 5 min of circulation compared to the CentriMag (Figure 6A). This trend remained through all time points.

vWF Immunoblotting

Immunoblotting indicates a gradual loss of high molecular weight (HMW) vWF multimers with increased pumping over time, consistent with reduced collagen binding activity observed in all pumps (Figure 6B).

Discussion

The CentriMag pump has a long successful history of clinical use (26) and we have demonstrated previously its consistent low blood damage profile during *in vitro* testing (19). Here we have used this as a baseline comparison for the HMII and HVAD which both produced significantly greater levels of blood damage for many of the tested parameters when compared to the CentriMag. Interestingly, the blood damage profiles of the HMII and the HVAD differed.

An increase in haemolysis as calculated by the amount of plasma-free haemoglobin was immediately evident after 60 min circulation in the HMII and increased substantially at the end of the 6 h test. In one study, patients implanted with the HMII (an axial flow pump) had higher levels of haemolysis than patients implanted with the VentrAssist centrifugal pump (27) . In order of least to most haemolysis was CentriMag < HVAD < HMII with none of the pumps affecting erythrocyte cell count. The same trend was observed with platelet activation with the HMII exhibiting the highest level after 360 min. Shared trends in haemolysis and platelet activation are suggestive of a link between the two. Singhal et al., demonstrated that extracellular haemoglobin activates platelets through binding to GP1Bα in a concentration-dependent manner (28) therefore the high levels of plasma-free haemoglobin in the HMII might be at least partially responsible for the higher levels of platelet activation. The HMII was compared to the HeartAssist-5 (HA-5) in a study by Chiu et al., using a mock circulatory loop and showed a 2.5 fold higher platelet activation than the HA-5 (29). This higher platelet activation was attributed to the recirculation and stagnant areas within the HMII leading to thrombus formation within the pump (30, 31).

Leukocytes play a role in both infection and thrombosis making them key elements in adverse events. Increased microparticle formation from platelets, leukocytes, and endothelial cells has been demonstrated clinically in VAD patients (32). These microparticles express anionic phospholipids, such as phosphotidylserine (PS), which are essential for initiating and propagating coagulation (33). PS can also induce endothelial damage in inflammatory environments (34). The trend for the emergence of LMPs differed to that for haemolysis and platelet activation with the order of least to most LMPs being: CentriMag < HMII < HVAD. The HVAD damaged the largest white blood cells, the granulocytes,

causing a significant reduction in granulocyte cell numbers and a significant increase in white blood cell microparticles as evidenced by an increase in "platelet numbers" (false positive) using the haematology analyser and by CD45-labelled microparticles using the flow cytometer. After 360 min circulation, the HVAD and HMII showed significantly higher numbers of $CD45^+$ LMPs ($p \leq 0.0001$). This is a new parameter and its clinical significance is unknown, but recent studies suggest high microparticle levels are associated with higher incidence of adverse events (33).

The leukocyte subset counts seem to correlate better with the activation status of the LMPs characterised as $CD11b^{Bright}HLADR^-$, $CD11b^{Bright}HLADR^+$, and $CD11b^{Dull}HLADR^+$. The $CD11b^{Bright}HLADR^-$ LMPs significantly increased after 240 min in the HMII and HVAD. The same trend for $CD11b^{Bright}HLADR^+$ was also apparent after 120 min. The $CD11b^{Bright}HLADR^-$ LMPs were positive for MM20A (Supplementary Figure 1), suggesting that these activated MPs are shed from granulocytes (35) and linked to the significant decrease in the granulocyte count in the HMII and HVAD. It is less clear if the $CD11b^{Bright}HLADR^+$ are shed from granulocytes but as the $CD11b^{Dull}HLADR^+$ lacked MM20A expression, they are unlikely to be granulocyte-derived. Woolley et al., investigated temporal leukocyte numbers and MAC-1 expression (a complement receptor consisting of CD11b and CD18) on granulocytes from patients implanted with HMII or HVAD. Leukocyte counts increased significantly at post-operative day 14 (POD14) but declined to below preoperative level at POD60 in both devices. MAC-1 expression on granulocytes was significantly higher in the HMII than the HVAD from POD14 through to POD60 (36) which correlate with the HMII having the highest number of $CD11b^{Bright}HLADR^-$ LMPs.

$CD11b^{Dull}HLADR^+$ LMPs showed a different trend, the number of these LMPs increased significantly from the static control after 120 min in all pumps, including the CentriMag. This suggests that the act of artificially pumping blood in a mock loop activates leukocytes no matter what the design. The cellular provenance of the $CD11b^{Dull}HLADR^+$ LMPs remains unknown. An increase in HLADR expression is a known marker of T cell activation (37) although the $CD11b^{Dull}HLADR^+$ LMPs were not positive for the bovine T cell marker MM1A (data not shown). The lack of expression of MM1A doesn't completely preclude these LMPs as being derived from T cells. There wasn't a significant decrease in lymphocyte

number with pumping but these LMPs could still be lymphocyte-derived, but arise from one of the minor subsets that might not manifest as a significant effect on total lymphocytes. Pulsatile-flow devices have shown a selective reduction in $CD4^+$ T cells, defective proliferative responses to stimuli such as staphylococcal enterotoxin B, and higher levels of apoptosis in $CD4^+$ and $CD8^+$ T cells in comparison to medically treated NYHA class IV patients (15, 16, 38, 39). An increase in the number of $CD11b^{Dull}HLADR^+$ LMPs in all three pumps tested here suggests that they are affected in continuous-flow devices. Recently, implanted continuous-flow VADs have been reported to increase the percentage T regulatory cells (Tregs) (40), which could increase LMP production.

All pumps showed a progressive decrease in vWF:CBA over time with no significant difference noted between the HVAD and HMII. We have previously shown that vWF degradation is influenced by varying degrees of shear stress (14). High levels of non-physiological shear stress activate ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) and unfold vWF multimers. Activated ADAMTS13 binds to and cleaves high molecular weight (HMW) vWF, however, excess cleavage results in enhanced vWF multimer degradation and reduced vWF activity (41, 42). Varying levels of shear stress in the CentriMag, HMII and HVAD could explain different collagen binding activity between devices. This observation is consistent with previous findings revealing that HMII and HVAD patients have similarly reduced vWF multimers and vWF activity, which might explain gastrointestinal bleeding episodes in HMII treated patients (17, 41). Notably, the HMII device generates higher levels of shear stress than the newer HeartMate 3 (HM3; Abbott, Thoratec, Pleasanton, CA, US) device. As a result, HMII patients show significantly increased HMW vWF degradation compared to HM3 patients (43). However, the MOMENTUM3 trial comparing the HMII with the HM3 showed no significant difference between the devices in terms of gastrointestinal bleeding events within six months post-implantation (44).

The HMII and HVAD have been used commonly in clinic since the mid-2000's after successfully completing animal studies and clinical trials (45, 46). Whilst both pumps were tested for haemolysis during development, in accordance with regulatory requirements neither was investigated thoroughly

in vitro for their total blood damage capabilities. Our study has shown the HMII causes substantial levels of haemolysis, increased platelet activation, high levels of LMPs, and a decrease in the binding activity of vWF. The latter has been studied *ex vivo* and our findings correlate with these. This study shows that blood circulated *in vitro* exhibits a decline in both HMW vWF multimers and vWF CBA. These findings are consistent with the enhanced vWF degradation profile and decreased CBA observed in HMII and HVAD patients (41, 47). Thus our more extensive *in vitro* results could relate to the prominence of thrombosis and bleeding identified in HMII and HVAD patients.

The HVAD performed slightly better than the HMII with regards to haemolysis and platelet activation, perhaps due to its substantially different design. However, the HVAD generated more $CD45^{+}$ LMPs, a significantly decreased granulocyte count, and an increased population of $MM20A^{+}CD11b^{Bright}HLADR^{-}$ MPs that are likely granulocyte-derived. These novel leukocyte assays used in our *in vitro* study reveals that the HVAD may be causing harm to the immune system through damaging granulocytes, the key cells for the removal of infectious pathogens. This is apparent clinically when comparing infection rates between the pumps - 35% in HVAD (48) and 23% in HMII (49) - and provides evidence that leukocyte analysis should be incorporated into VAD development.

A key limitation of this study which must be considered when interpreting our findings is that these pumps were not as originally manufactured. However, it is clear that assessing the total blood damage capability of VADs prior to implantation provides a wealth of knowledge that may be beneficial and perhaps predict clinical outcomes.

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Figure legends

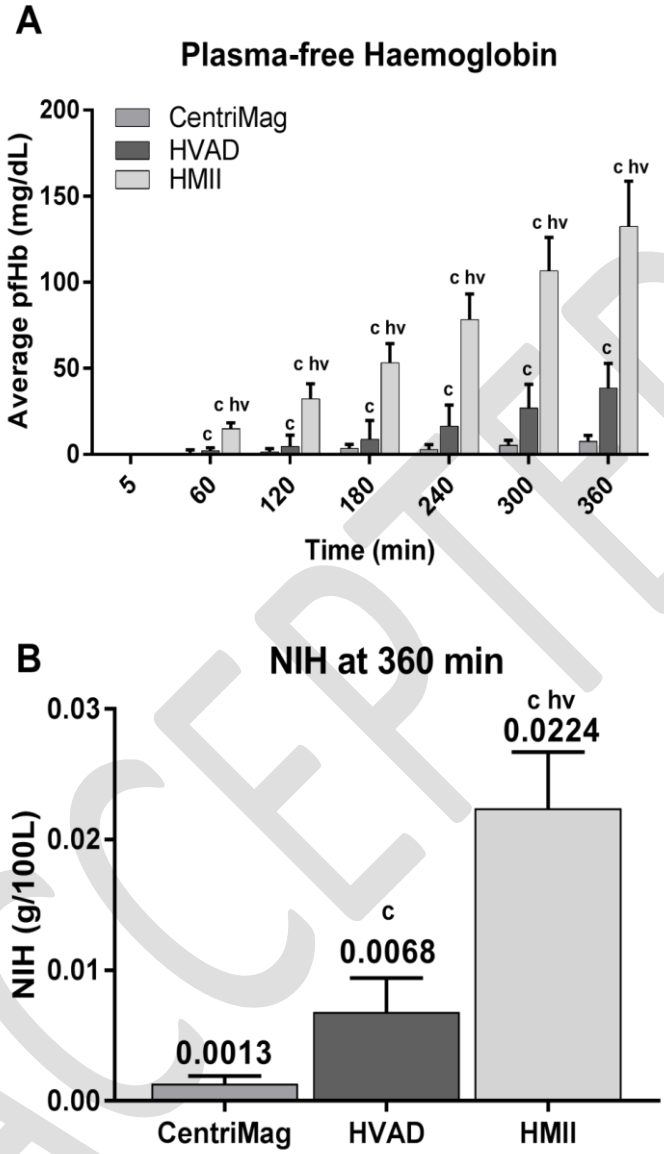


Figure 1: Haemolysis generated in the CentriMag, HeartMate 2, and HVAD

Bovine blood diluted to a haematocrit of 30 ± 2% using PBS was loaded into the mock circulatory loops for the CentriMag (n = 25), HeartMate 2 (HMII; n = 12), and HVAD (n = 11). A 500 mL bag of bovine blood was left in the +37°C water bath as a static control (n = 25). Blood samples were removed every hour for 6 hours. **A**. Average plasma-free haemoglobin (pfHb; mg/dL) levels over time. **B.** Normalised Index of Haemolysis (NIH; g/100L) at 360 min. Mean ± SD; c = significantly different from the CentriMag ($p < 0.05$); hv = significantly different from the HVAD ($p < 0.05$).

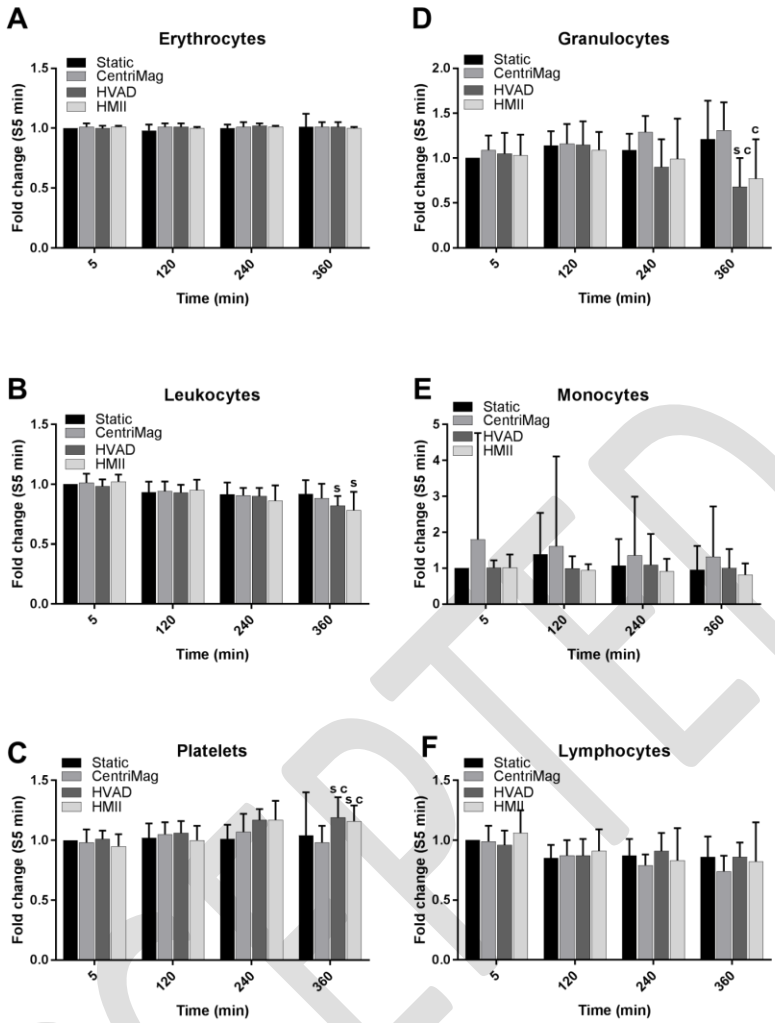


Figure 2: Haematology for the CentriMag, HeartMate 2, and HVAD

Bovine blood diluted to a haematocrit of 30 ± 2% using PBS was loaded into the mock circulatory loops for the CentriMag (n = 25), HeartMate 2 (HMII; n = 12), and HVAD (n = 11). A 500 mL bag of bovine blood was left in the +37°C water bath as a static control (n = 25). Blood samples were removed every hour for 6 hours. Average complete cell counts from blood samples run in triplicate were acquired on a veterinary haematology analyser. Counts displayed as a fold change from the static control at 5 min. **A.** Erythrocytes; **B.** Leukocytes; **C.** Platelets; **D.** Granulocytes; **E.** Monocytes; **F.** Lymphocytes. Mean ± SD; s = significantly different from the static control ($p < 0.05$); c = significantly different from the CentriMag ($p < 0.05$).

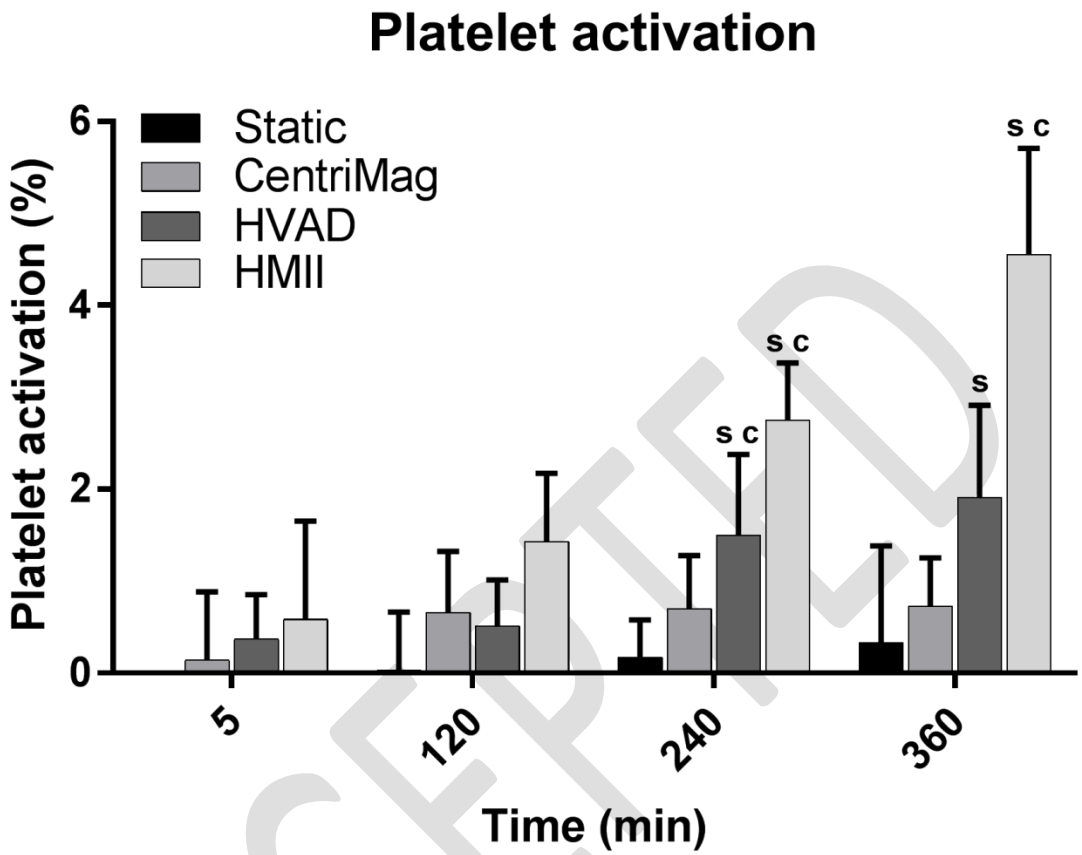


Figure 3: Platelet activation for the CentriMag, HeartMate 2, and HVAD

Bovine blood diluted to a haematocrit of 30 ± 2% using PBS was loaded into the mock circulatory loops for the CentriMag (n = 17), HeartMate 2 (HMII; n = 8), and HVAD (n = 10). A 500 mL bag of bovine blood was left in the +37°C water bath as a static control (n = 19). Blood samples were removed every hour for 6 hours. Platelet activation (%) was measured over time using flow cytometry. Mean ± SD; s = significantly different from the static control ($p < 0.05$); c = significantly different from the CentriMag ($p < 0.05$).

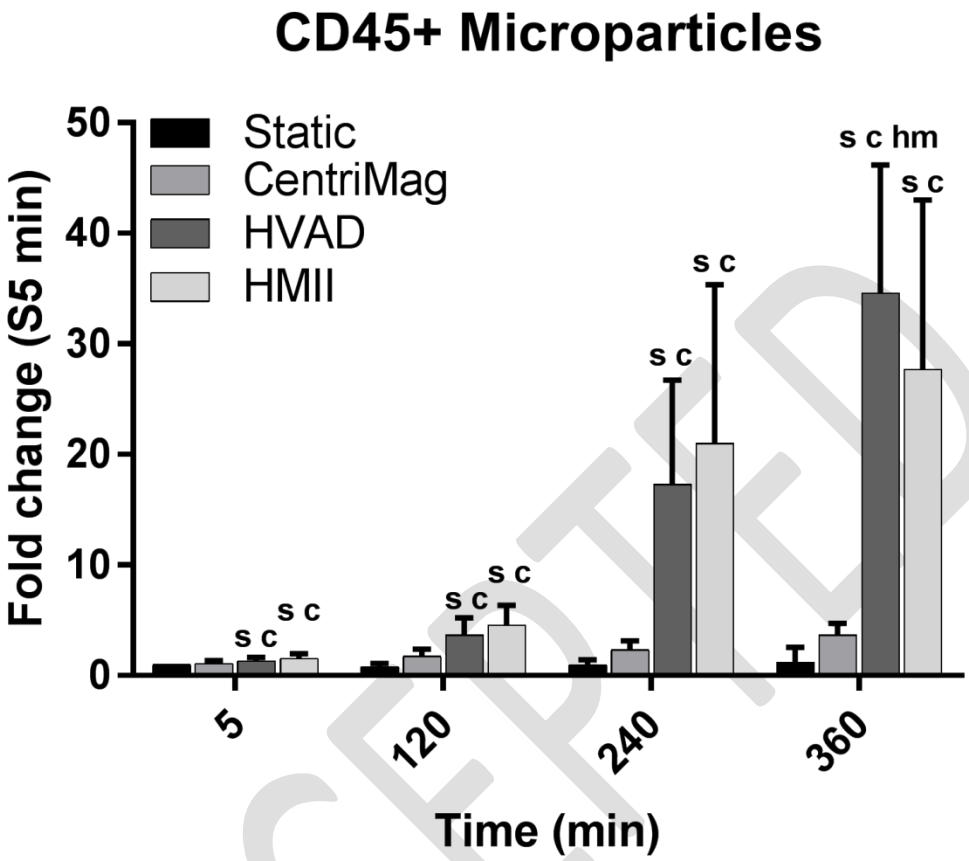


Figure 4: Leukocyte-derived microparticles in the CentriMag, HeartMate 2, and HVAD

Bovine blood diluted to a haematocrit of 30 ± 2% using PBS was loaded into the mock circulatory loops for the CentriMag (n = 15), HeartMate 2 (HMII; n = 10), and HVAD (n = 9). A 500 mL bag of bovine blood was left in the +37°C water bath as a static control (n = 15). Blood samples were removed every hour for 6 hours. Number of CD45-positive ($CD45^+$) events with a low side-scatter was measured over time using flow cytometry. Mean ± SD; s = significantly different from the static control ($p < 0.05$); c = significantly different from the CentriMag ($p < 0.05$); hm = significantly different from the HeartMate 2 ($p < 0.05$).

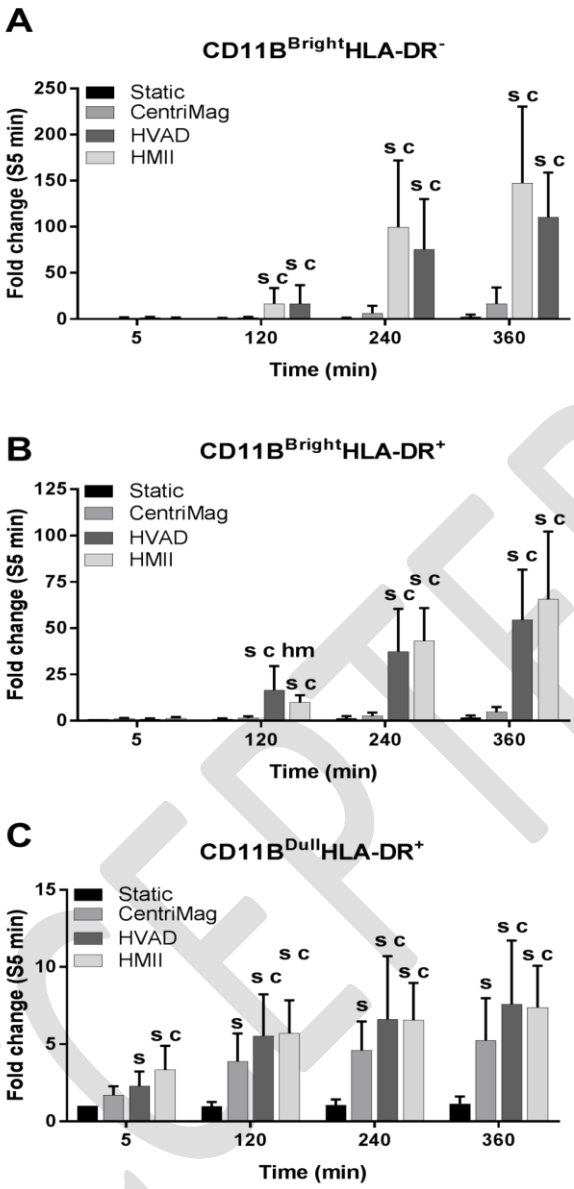


Figure 5: Microparticles expressing activation markers in the CentriMag, HeartMate 2, and HVAD

Bovine blood was diluted to a haematocrit of 30 ± 2% using PBS, loaded into the loops with the CentriMag (n = 13), HeartMate 2 (HMII; n = 9), and HVAD (n = 8). Blood from 5, 120, 240, and 360 min were stained with MM20A, MM1A, CD14, CD21, CD11b, and HLA-DR and the number of microparticles with the following expression: **A.** $CD11b^{Bright}HLA\text{-}DR^{-}$, **B.** $CD11b^{Bright}HLA\text{-}DR^{+}$, and **C.** $CD11b^{Dull}HLA\text{-}DR^{+}$, plotted for each pump type as a fold from the 5 min static control. Mean ± SD; s = significantly different from the static control ($p < 0.05$); c = significantly different from the CentriMag ($p < 0.05$).

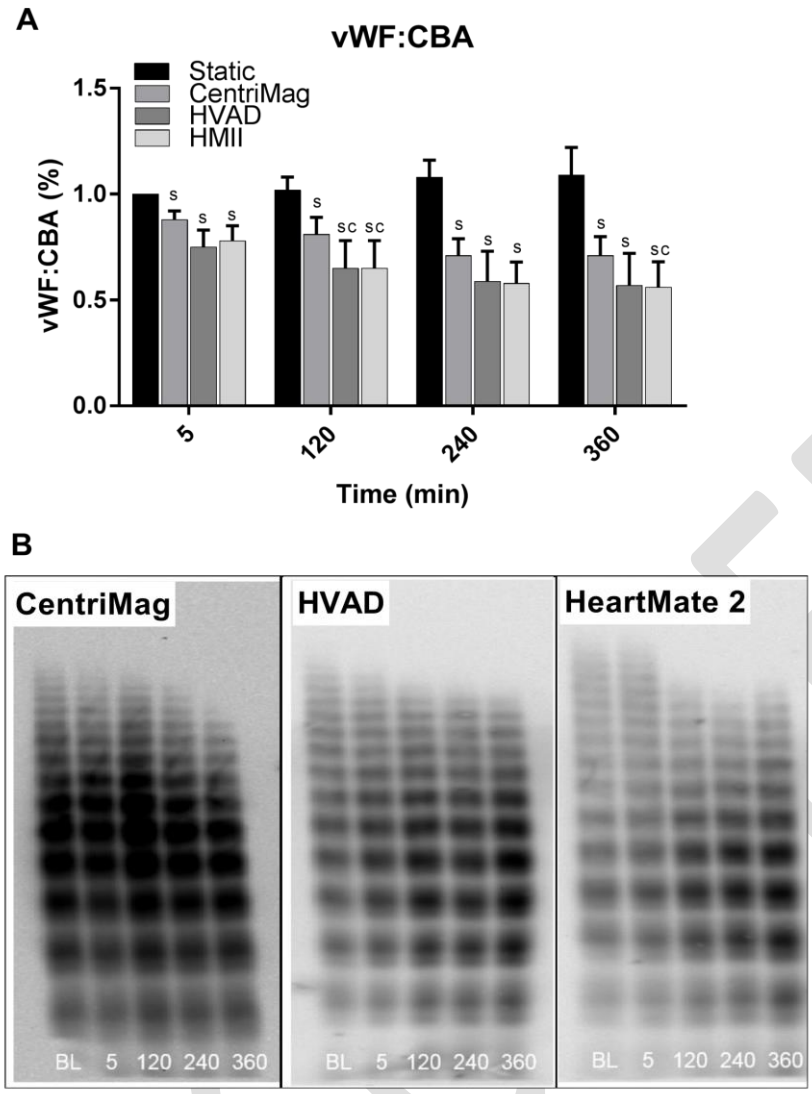
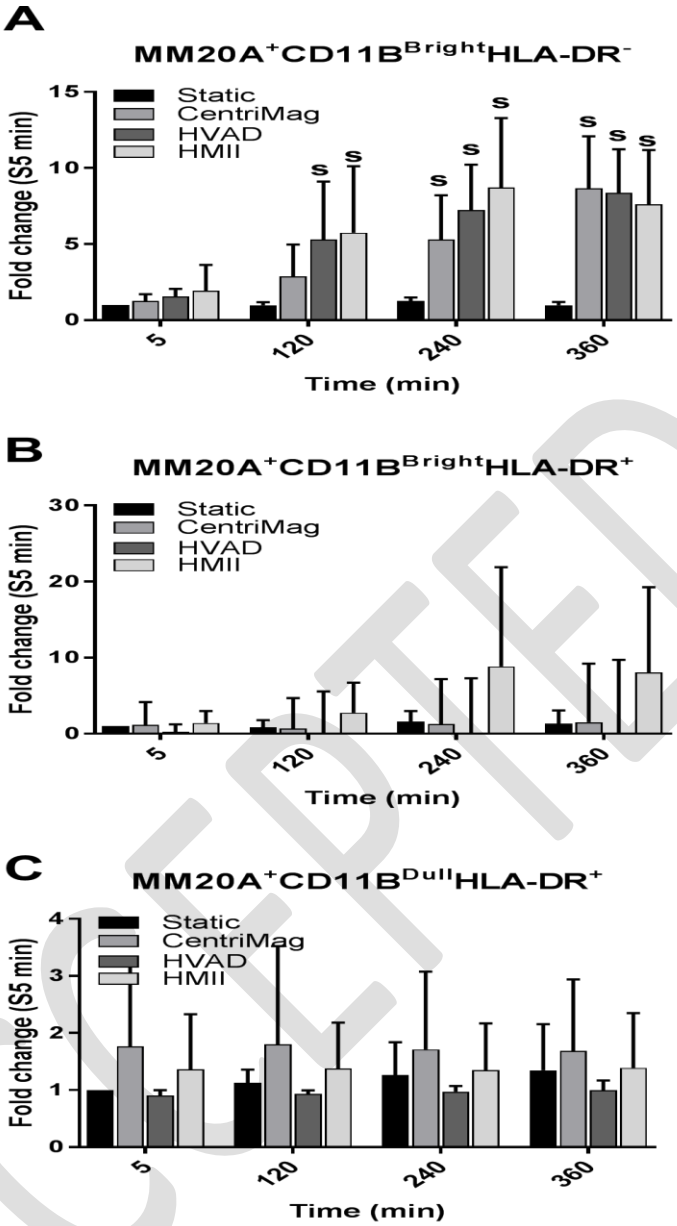


Figure 6: von Willebrand Factor degradation in the CentriMag, HeartMate 2, and HVAD

Blood from 5, 120, 240, and 360 min was centrifuged to obtain platelet-poor plasma (PPP). Samples were analysed for von Willebrand Factor Collagen Binding Activity (vWF:CBA) using ELISA; CentriMag (n = 8), HeartMate 2 (HMII; n = 6), and HVAD (n = 8). Immunoblots revealed degradation of high molecular weight vWF (HMW:vWF). **A.** The collagen binding activity (CBA) of vWF over time in each pump. **B.** A representative immunoblot image of HMW:vWF multimer degradation. Mean ± SD; s = significantly different from the static control ($p < 0.05$); c = significantly different from the CentriMag ($p < 0.05$).



Supplementary Figure 1: MM20A expression on activated microparticles

Expression of the granulocyte marker MM20A on activated microparticles as a fold change from the static control at 5 min over time. MM20A expression on the following populations: **A.** $CD11b^{Bright}HLA\text{-}DR^{-}$, **B.** $CD11b^{Bright}HLA\text{-}DR^{+}$, and **C.** $CD11b^{Dull}HLA\text{-}DR^{+}$. Mean ± SD; s = significantly different from the static control ($p < 0.05$); c = significantly different from the CentriMag ($p < 0.05$).