Circulating microparticles are increased amongst people presenting with HIV and advanced immune suppression in Malawi and correlate closely with arterial stiffness: a nested case control study [version 1; peer review: 1 approved with reservations]

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Abstract
Background: We aimed to investigate whether circulating microparticle (CMPs) subsets were raised amongst people presenting with human immunodeficiency virus (HIV) and advanced immune suppression in Malawi, and whether they associated with arterial stiffness.
Methods: Antiretroviral therapy (ART)-naïve adults with a new HIV diagnosis and CD4 <100 cells/µL had microparticle characterisation and carotid femoral Pulse Wave Velocity (cfPWV) at 2 weeks post ART initiation. HIV uninfected controls were matched on age, systolic blood pressure (BP) and diastolic BP in a 1:1 ratio. Circulating microparticles were identified from platelet poor plasma and stained for endothelial, leucocyte, monocyte and platelet markers.
Results: The median (IQ) total CMP count for 71 participants was 1 log higher in HIV compared to those without (p<0.0001) and was associated with arterial stiffness (spearman rho 0.47, p<0.001). In adjusted analysis, every log increase in circulating particles showed a 20% increase in cfPWV (95% confidence interval [CI] 4 – 40%, p=0.02).
In terms of subsets, endothelial and platelet derived microparticles were most strongly associated with HIV. Endothelial derived E-selectin+ CMPs were 1.3log-fold higher and platelet derived CD42a+ CMPs were 1.4log-fold higher (both p<0.0001). Endothelial and platelet derived CMPs also correlated most closely with arterial stiffness (Spearman rho: E-selectin+ 0.57 and CD42a 0.56, both p<0.0001).

**Conclusions:** Circulating microparticles associate strongly with arterial stiffness among people living with HIV in Malawi. Endothelial damage and platelet microparticles are the predominant cell origin types and future translational studies could consider prioritising these pathways.

**Keywords**
HIV, microparticles, cardiovascular

This article is included in the Malawi-Liverpool Wellcome Trust Clinical Research Programme gateway.
Introduction
People living with human immunodeficiency virus (HIV) (PLWH) are at increased risk of cardiovascular diseases\(^1\). Heightened inflammation has been implicated in vascular dysfunction but the underlying pathogenetic mechanisms have not been fully elucidated\(^2\). These mechanisms are even more complex in the low-income sub-Saharan Africa setting (SSA), where additional factors including latent or recurrent infections, late presentation and antiretroviral therapy (ART) failure are prevalent\(^3\). In Malawi, several studies have shown an increased risk of stroke associated with HIV\(^4\).

The first few months of ART carry a particularly high cardiovascular risk. In a case control study of 705 patients with stroke, Benjamin et al found an adjusted odds ratio of 15.6 among those within the first 6 months of ART compared to those not yet started on treatment and 12.9 among those with a CD4 count of less than 200 cells/mm\(^3\) compared to those with CD4 greater than 500 cells/mm\(^3\). Further, we have previously shown that amongst people presenting with HIV and a CD4 count less than 100 cells/mm\(^3\), arterial stiffness is increased by 12% (adjusted fold change) compared to healthy volunteers living without HIV\(^5\). Arterial stiffness has been used as a measure of vascular dysfunction in HIV studies as well as other chronic inflammatory conditions\(^6,7\) and we have also previously shown that arterial stiffness, as measured by carotid femoral pulse wave velocity (cfPWV), is increased in people experiencing unstructured treatment interruption\(^8\). cfPWV is a gold standard measurement of arterial stiffness and has been validated as a physiological biomarker of cardiovascular events and mortality\(^9,10\).

The increase in arterial stiffness previously identified only persists during the first 3 months of ART and is associated with markers of inflammation, including T cell activation\(^1\). Given the timing of this inflammatory process, it is tempting to hypothesise that increased cardiovascular risk during this time may follow a similar process to immune reconstitution inflammatory syndrome (IRIS). During the normal inflammatory response leucocytes adhere to the endothelial cells via vascular cell adhesion molecule (VCAM) and intracellular adhesion molecule (ICAM), and undergo transcytosis leading to the development of foam cells\(^11\). Pro-thrombotic pathways attract platelets to the resulting plaque and subsequent migration of smooth muscle cells into the intima leads to the formation of a fibrous cap\(^12\). During this process, elastin is degraded by enzymes such as matrix metalloproteinase (MMP) and is replaced by collagen, increasing arterial stiffness\(^13\).

Circulating microparticles (CMPs) are released into the circulation following activation or apoptosis of the affected cells\(^14\). Through a process of bleeding, microparticles are formed from the originating host cell’s outer membrane (e.g. leucocytes, platelets and endothelial cells). During this process annexin V molecules, which are normally located on the inner membrane of a cell, are flipped round to become expressed on the microparticle surface\(^15\). These microparticles also express the markers expressed on the surface of the cell of origin and so microparticle subsets are an indication of which cells are undergoing stress.

Previous studies in HIV have assessed the presence and function of CMPs amongst PLWH in high resource settings and have separately highlighted tissue factor expression, imbalanced endothelial progenitor cell proportions and platelet activation\(^16-22\). Hijmans et al recently demonstrated evidence of in vitro endothelial cell stress, apoptosis and senescence induced by leucocyte, platelet and endothelial microparticle subsets from patients with HIV\(^23\). Here, we aim to take the characterisation of circulating microparticles in HIV a step further by assessing their relationship with cardiovascular risk and seeking to elucidate pathways that might be involved in heightened inflammation during early ART.

Methods
Ethical considerations
All participants provided informed written consent and ethical approval was granted by the College of Medicine Research and Ethics Committee (COMREC), University of Malawi (P.09/13/1464) and the University of Liverpool Research and Ethics Committee (UoL000996).

Study cohort
The Study into HIV, Immune activation and Endothelial Dysfunction (SHIELD) cohort recruited 279 ART-naïve adults with a new HIV diagnosis and CD4 <100 cells/µL from the ART clinic and voluntary HIV testing clinic at Queen Elizabeth Central Hospital, Blantyre, Malawi, along with 110 adults without HIV infection and without evidence of infection within the previous 3 months as determined by the study clinician. This cohort was recruited between January 2014 and June 2015 and has been reported on previously, and full details of recruitment and methods are available in 5. In brief, participants underwent a detailed clinical assessment, blood draw for plasma storage and cfPWV at 2 weeks post ART initiation. cfPWV measured arterial stiffness using a Vicorder device (Skidmore Medical, London, UK) according to standardised guidelines\(^24\). A random sample of wave forms was reviewed by an experienced independent assessor at three timepoints during the study to ensure consistent quality.

SHIELD participants with HIV infection were ordered in a continuous fashion according to cfPWV values from lowest to highest. A convenience sample of 36 was chosen across the spectrum of cfPWV values in two groups. Firstly, we selected participants from the group with a cfPWV in the highest quartile (>8.2 m/s). We selected 24 of these participants so that there was an even distribution across the range of values. Secondly, we chose participants from those below the highest quartile. We chose 12 of these participants again so that there was an even distribution across the range of values. This approach aimed to enrich the number of potential CMPs for subset analysis (we hypothesised they would be higher at higher ranges of cfPWV) whilst also capturing a range of values to analyse associations between total CMP count and cfPWV.
Participants without HIV were then matched to the selected participants with HIV infection on age, systolic blood pressure (BP) and diastolic BP in a 1:1 ratio.

Statistical analysis

Wilcoxon rank-sum and spearman rho tests were used to test the association between CMPs and categorical or continuous variables, respectively. To allow for multiple comparisons characterising 18 types of CMPs, the Bonferroni correction was applied and a p value of less than 0.003 was used as significant. Linear regression was used to examine the association between total CMPs on cfPWV which was log transformed for normality. The model was adjusted for confounders (age and sex) and mediators (blood pressure, haemoglobin and HIV) as identified previously. Analysis was carried out on STATA version 13.1.

Laboratory procedures

**Microparticle isolation.** Plasma samples frozen at -80°C were thawed in a 37°C water bath for 1 minute. 250µL was centrifuged at 5000g for 5 minutes in order to isolate platelet poor plasma (PPP). PPP was centrifuged at 16000g for 60 minutes and the PPP was decanted to leave 20µL of microparticle pellet. Distilled water was filtered through 0.22 µm syringe filter under a flow hood was added to Annexin V 10x buffer at a 1:10 dilution. Annexin V 1x buffer was added to the microparticle pellet to a volume sufficient to allow 35µL of microparticle/Annexin V buffer solution for each antibody combination being tested as well as controls.

**Microparticle staining.** Each antibody was diluted to a 1:100 concentration in either AnV buffer for AnV antibodies or in phosphate-buffered saline (PBS) for all remaining antibodies. 5µL of AnV antibody was added to each well containing 35µL of microparticle AnV buffer solution. The remaining origin stains were then added at a volume of 10µL for those tubes that only had one origin stain and 5µL for those tubes that had two origin stains. This was to ensure a total staining volume of 50µL for all samples. Single stain samples were also acquired for the purposes of compensation and isotype controls were analysed for the purposes of gating. For the isotype controls, 10µL of 1:40 isotype control antibody was added to the 35µL microparticle AnV buffer solution along with 5µL of AnV antibody (IgG1 PE, R&D Systems; IgG1k PE, IgG1 FITC, R&D Systems; IgG1k APC Cy7, BD Pharmingen; IgGMk PE, BD Pharmingen).

Following staining plates were covered with foil and agitated at room temperature for 20 minutes. 200µL AnV buffer was added to every well and then transferred to FACS tubes. A further 400µL AnV buffer was then added to every tube. Finally, 6µL of 3µm latex beads (SIGMA) were added to 2ml of distilled filtered water and 10µL of that was added to 650µL distilled filtered water to allow microparticle enumeration. Beads size 1.1 µm were used for microparticle gating, with microparticles 1 µm or smaller and expressing AnnexinV being categorised as microparticles.

Endothelial, leucocyte, monocyte and platelet microparticles were chosen as common circulating microparticles involved in inflammation and were identified by flow cytometry on a CyAn ADP 9 colour flow cytometer (Beckman Coulter). FITC stained for Annexin V (BD Pharmingen), PE stained for VCAM, ICAM, E-selectin, 66b or CD16 (BD Pharmingen) and APC-Cy7 stained for PECAM or CD14 (BD Pharmingen). To identify tissue factor expression on monocytes, PE stained Annexin V, APC-Cy7 stained CD14 (BD Pharmingen) and FITC stained tissue factor (Sekisui Diagnostics). Single stain samples were also acquired for the purposes of compensation and isotype controls were analysed for gating.

**Microparticle gating.** A microparticle protocol was created on the CyAn flow cytometer with the same voltage settings as the T cell and monocyte panels but with a lower capture threshold of 0.01% instead of 2%. This was to ensure that microparticles were not excluded as debris. 350µL of each FACS tube was acquired and the plots were then transferred to FlowJo (Tree star Inc.) for analysis. After identification of singlets, the microparticle pellet was gated on forward scatter and AnV (FITC) to identify the microparticle population which was less than 1µm in size and expressing AnV. Gates were applied using thresholds provided by isotype controls (see Figure 1).

**Cell surface immunophenotyping.** Cell surface immune phenotyping was carried out for CD4 and CD8 T cell activation (HLA-DR+/CD38), exhaustion (PD1+) and senescence (CD57+), as well as monocyte subsets (classical CD14+CD16-, intermediate CD14+CD16+ and nonclassical CD14+CD16+) as described previously. The T-cell panel consisted of CD3 BV510, CD4 V450, CD38 PE Cy7, BD Pharmingen, HLA-DR AF700, PD1 APC, and CD57 FITC (all from BD Biosciences) and CD8 PE (Biolegend). The monocyte panel consisted of HLA-DR AF700, CD14 PE Cy7 and CD16 PE (all from BD Biosciences).

**Results**

CMP data were available for 33 PLWH and 36 people without HIV. Sufficient plasma samples were not available for three of the selected participants with HIV. For the 69 participants with available CMP data, median (interquartile range [IQR]) age was 41 years (35 – 49) and 28 (41%) were female. In total, 31 (45%) had an education level of at least primary school completion. Median (IQR) blood pressure was 130/78 (118/70 – 134/80) and 11 (16%) had a history of smoking. Characteristics according to HIV status are shown in Table 1 (for in depth comparison according to HIV status see 5). For the 33 PLWH, median (IQR) CD4 and HIV viral load were 42 cells/µL (31 – 71) and 1.1x10⁵ copies/mL (0.4 – 2.6) respectively.

**Relationship between absolute CMP counts, HIV and arterial stiffness**

For the overall cohort, the median (IQ) total CMP count was approximately 1 log higher in participants with HIV compared to those without (Table 1). Total CMP counts were also significantly associated with arterial stiffness (spearman rho
Figure 1. Identification of the microparticle population and subset gating strategy. (A) 1.1µm beads were used to find forward scatter gate for microparticle identification. (B) Microparticle pellet was obtained from platelet poor plasma and stained for Annexin V. Total circulating microparticle count was measured by identifying microparticles Annexin V positive and less than 1.1µm forward scatter as shown. (C) Total circulating microparticles in a patient with a high count compared to B. (D) Isotope controls identified gates for PE and APC-Cy7 stains for each subset panel. (E) Leucocyte microparticles. (F) Endothelial or platelet microparticles.

0.47, p<0.001; Figure 2), as well as faster heart rate and higher creatinine (spearman rho: 0.31; p=0.01 and 0.28; p=0.02). When adjusted for a priori identified mediators and confounders (age, sex, haemoglobin and blood pressure), higher log total CMPs were associated with an increased cPWV [fold change 1.20m/s, 95% confidence interval (CI) 1.04 – 1.40; p=0.02]. When additionally adjusted for HIV status, the association between log total circulating microparticle count and cPWV remained similar [fold change 1.07m/s (95% CI 1.23 – 1.01), p=0.046; Table 2].

Total CMP counts also correlated directly with proportions of CD4 and CD8 T cells expressing markers of activation, exhaustion and senescence [spearman rho (p value): CD8 activation 0.49 (0.0004), exhaustion 0.39 (0.041), senescence 0.44 (0.0006); CD4 activation 0.44 (0.071), exhaustion 0.27 (0.0007), senescence 0.44 (0.01)]. However, there was no association between total CMPs and monocyte subsets [spearman rho (p value): classical 0.08 (0.57), intermediate -0.018 (0.90), nonclassical -0.07 (0.63)].

Relationship between CMP subsets, HIV and arterial stiffness

The largest elevations in CMP subsets amongst PLWH were seen with endothelial and platelet derived microparticles. Endothelial derived E-selectin+ CMPs were 1.3-fold higher [median (IQR) 13.0 log particles/mL (12.5 – 14.3) vs 9.9 (8.9 – 10.5); p<0.0001]. The increase in CMPs expressing PECAM but not E-selectin was less significant (Figure 3). Platelet derived CD42a+ CMPs were 1.4-fold higher amongst PLWH [median (IQR) CD42a 15.1 log particles/mL (13.7 – 16.3) vs 10.5 (9.4 – 11.6); p<0.0001]. Amongst leucocyte derived CMPs,
Table 1. Clinical characteristics of 67 SHIELD participants with microparticle data according to human immunodeficiency virus (HIV) status.

<table>
<thead>
<tr>
<th></th>
<th>People living with HIV n=33</th>
<th>People living without HIV n=36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median or Frequency</td>
<td>IQR or %</td>
</tr>
<tr>
<td>Age (years)</td>
<td>41</td>
<td>38 - 50</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>39%</td>
</tr>
<tr>
<td>Primary school education or less</td>
<td>16</td>
<td>48%</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>21.7</td>
<td>19.4 – 24.7</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>130</td>
<td>122 – 135</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>80</td>
<td>74 – 88</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>12</td>
<td>11 – 13</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.3</td>
<td>3.7 – 4.7</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>4.6</td>
<td>4.3 – 5.0</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>68</td>
<td>58 – 83</td>
</tr>
<tr>
<td>History of ever smoking</td>
<td>6</td>
<td>18%</td>
</tr>
<tr>
<td>History of a diagnosed cardiovascular disease</td>
<td>3</td>
<td>9%</td>
</tr>
<tr>
<td>Co-infection at time of recruitment*</td>
<td>3</td>
<td>9%</td>
</tr>
<tr>
<td>CD4 count (cells/µL)</td>
<td>42</td>
<td>31 - 71</td>
</tr>
<tr>
<td>HIV Viral Load (x10⁵ copies/mL)</td>
<td>1.1</td>
<td>0.4 – 2.6</td>
</tr>
<tr>
<td>cfPWV (m/s)</td>
<td>9.1</td>
<td>8.2 – 10.3</td>
</tr>
<tr>
<td>Total Circulating Microparticle Count (log particle/mL)</td>
<td>6.7</td>
<td>6.3 – 7.3</td>
</tr>
</tbody>
</table>

*Acute co-infection, refers to diagnosis of an infection other than HIV. BP=blood pressure.

Figure 2. Association between total circulating microparticles and carotid femoral pulse wave velocity (m/s). CI=confidence interval.
Table 2. Linear regression analysis showing the association between total circulating microparticles and carotid femoral Pulse Wave Velocity.

<table>
<thead>
<tr>
<th></th>
<th>Association between total circulating microparticle count and cfPWV</th>
<th>Association between total circulating microparticle count and cfPWV adjusting for HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold change</td>
<td>95% CI</td>
</tr>
<tr>
<td>Total Microparticle Count (per log particle/mL increase)</td>
<td>1.20</td>
<td>1.04 – 1.40</td>
</tr>
<tr>
<td>Age (per 10-year increase)</td>
<td>1.14</td>
<td>1.03 – 1.26</td>
</tr>
<tr>
<td>Female sex</td>
<td>0.85</td>
<td>0.67 – 1.07</td>
</tr>
<tr>
<td>Diastolic Blood pressure (per 10mmHg increase)</td>
<td>1.20</td>
<td>1.06 – 1.38</td>
</tr>
<tr>
<td>Haemoglobin (per g/dL increase)</td>
<td>1.04</td>
<td>0.98 – 1.10</td>
</tr>
<tr>
<td>HIV infection</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A multivariate model was constructed to assess the association between circulating microparticles (CMPs) and carotid femoral Pulse Wave Velocity (cfPWV) using data from patients with and without HIV combined. The models are adjusted for mediators and confounders as identified from previous work in this cohort. The first model provides the association between CMPs and arterial stiffness adjusting for mediators and confounders but excluding HIV. The second model adds HIV to assess for any role of HIV in mediating the association between CMPs and arterial stiffness. HIV=human immunodeficiency virus; CI=confidence interval.

Figure 3. Microparticle subsets according to human immunodeficiency virus (HIV) status for 69 Malawian adults. PLWH=People living with HIV. No HIV= People living without HIV. Red lines represent median microparticles count. Grey Asterix represent p value: ns denotes p>0.003, * denotes p<0.003 but >0.0001, **denotes p<0.0001, ***denotes p<0.0001. TF=tissue factor. CD=cluster of differentiation. VCAM=vascular cell adhesion molecule 1. ICAM=intracellular cell adhesion molecule 1. PECAM=platelet endothelial cell adhesion molecule.
CD66b+ and CD16+ CD14- were 1.4-fold and 1.5-fold higher respectively [median (IQR) CD66b: 11.9 log particles/mL (11.3 – 12.7) versus 8.8 (7.9 – 9.3), p<0.0001. CD16+CD14-: 13.1 (11.3 – 133.9) vs 8.7 (7.4 – 9.1), p<0.0001]. For CD14 positive CMPs, only those expressing tissue factor were significantly higher [1.4-fold; median (IQR) 10.1 log particles/mL (8.3 – 11.3) vs 7.0 (6.2 – 8.1), p<0.0001].

CMPs of endothelial and platelet origin associated closely with cfPWV [spearman rho: E-selectin+ 0.57, p<0.0001 and CD42a 0.56, p<0.0001; Table 3]. CD16 positive CD14 negative CMPs also correlated significantly with cfPWV (spearman rho 0.69, p<0.0001).

**Discussion**

Here we show that total CMPs correlate closely with arterial stiffness and are markedly increased amongst people who present with HIV and advanced immune suppression in Malawi. Microparticles originating from endothelium and platelets were especially high amongst people with HIV and correlated strongly with arterial stiffness; suggesting that these pathways might be prioritised for future studies seeking to reduce inflammation driven cardiovascular risk amongst PLWH during ART initiation.

Each log increase in CMPs showed a 20% increase in cfPWV. A relationship between total CMPs and activated T cells was also demonstrated, in keeping with our previous data suggesting a role for activated and exhausted T cells in increased arterial stiffness amongst PLWH. However, the association between CMPs and arterial stiffness was not reduced when HIV was added, suggesting that other factors may be at play in the generation of CMPs in this Malawian population.

CMPs are induced in response to cellular stress in the form of activation, apoptosis or physical shear stress. A similar case control study of 15 PLWH established on ART in the USA, showed significantly elevated levels of all types of microparticles compared to 15 participants without HIV infection, where microparticles from PLWH directly impaired endothelial cell function. A larger study of PLWH from the Czech Republic also found an increase in microparticles compared to participants without HIV, but found no difference amongst those on ART compared to those who were at their first presentation. However, none of these studies assessed the relationship between CMPs and arterial stiffness. By presenting associations with a validated physiological biomarker, we provide evidence for the use of CMPs as a potential clinically relevant tool to support characterisation of inflammation amongst PLWH.

As well as acting as biomarkers of inflammation, microparticles have been implicated in mediating inflammation induced pathogenesis. They may interact with endothelial cell surface adhesion molecules and cause endothelial damage through the production of nitrous oxide and pro-inflammatory cytokines. CMPs have been shown to transfer important material such as miRNA and lipids to other cells. In particular, the transfer of the CCR5 receptor to endothelial cells from leucocyte

<table>
<thead>
<tr>
<th>Circulating Microparticle subset</th>
<th>Spearman’s rho</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>Endothelial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PECAM+CD42a-</td>
<td>0.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Eselectin+</td>
<td>0.61</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PECAM+Eselectin-</td>
<td>-0.34</td>
<td>0.004</td>
</tr>
<tr>
<td>PECAM+Eselectin+</td>
<td>0.55</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PECAM-Eselectin+</td>
<td>0.53</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ICAM+PECAM+</td>
<td>0.33</td>
<td>0.02</td>
</tr>
<tr>
<td>ICAM+PECAM-</td>
<td>0.58</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VCAM+PECAM+</td>
<td>0.26</td>
<td>0.07</td>
</tr>
<tr>
<td>VCAM+PECAM-</td>
<td>0.46</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Leucocyte</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD66b+PECAM-</td>
<td>0.39</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD14-CD16+</td>
<td>0.74</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD14+CD16+</td>
<td>0.08</td>
<td>0.78</td>
</tr>
<tr>
<td>CD14+CD16-</td>
<td>-0.06</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>Platelet</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD42a+PECAM-</td>
<td>0.61</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
derived microparticles potentially renders them permissible to
direct HIV infection\(^\text{19}\). Circulating microparticles pro-
duced in response to HIV could therefore lead to endothelial
damage, and therefore increased risk of cardiovascular disease,
through multiple mechanisms including direct endothelial adhe-
sion and activation, transfer of cytotoxic viral proteins, and
propagation of low-level viral replication.

We discovered that microparticles of endothelial and plate-
let origin are particularly expanded amongst PLWH in this
low income SSA setting. Defining endothelial micropartic-
cles as CD51+, da Silva et al previously reported endothelial
microparticles 20 times higher in PLWH compared to people
without HIV\(^\text{19}\). Endothelial microparticles promote expression
of adhesion molecules on the endothelial cell surface, generating
thrombosis, platelet activation and recruitment of inflam-
matory cells\(^\text{12}\). Platelet driven thrombosis in combination
with endothelial activation may take advantage of a pro-
aimmed endothelial barrier and lead to decreased elasticity and
smooth muscle reactivity within the arterial wall\(^\text{11}\). Inflammatory
cytokine release in response to ongoing cellular recruit-
ment, as well as from direct effects of the HIV virus, can also
activate and increase the proliferation and migration of vas-
cular smooth muscle cells, and thus induce arterial stiffness\(^\text{13}\).
Wheway et al. have previously found endothelial microparticles
to form conjugates with T cell subsets, with increased binding
to those cells that were pre-stimulated\(^\text{1}\). Binding was VCAM
and ICAM dependent and endothelial microparticles were able to
stimulate in vitro proliferation of T cells.

Although this study examines a small convenience sample, we
demonstrate quantification and characterisation of circulat-
ing microparticles to be clinically relevant and also confirm
that this applies in the low-income sub-Saharan Africa setting.
We also elucidate the platelet – endothelial axis as an interest-
ing pathway worthy of further investigation in inflammation
during early ART. This cohort of people living with HIV had
experienced advanced immune suppression and so factors other
than the direct effect of HIV itself (e.g. cytomegalovirus, tuber-
culosis, cryptococcal disease) may be contributing. Further,
findings are not generalisable to cohorts with more robust CD4
counts and therefore this needs to be established.

Overall, the characterisation of microparticles in this study
lends weight to a model where active and significant immune
activation amongst people living with HIV is strongly related
to endothelial damage and may involve both CD8 T cell and
platelet activation. Further research should investigate
whether CMPs might represent translational targets to reduce
inflammation driven cardiovascular risk amongst people living
with HIV.

Data availability
Underlying data
OPENICPSR: Circulating Microparticles are increased amongst
people presenting with HIV and advanced immune suppress-
ion in Malawi and correlate closely with arterial stiffness.
https://doi.org/10.3886/E147761V2\(^\text{15}\).

This project contains all underlying and extended data (study
materials) for this study. Due to the sensitive nature of this
project, these data are hosted on a data host specialising in stig-
matised disorders and an account can be created for free to
access the data.

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their support with study design and implementation. An earlier
version of this article can be found on medRxiv (doi: https://doi.

References
of non-AIDS causes of death among HIV-infected individuals receiving
antiretroviral therapy: a systematic review and meta-analysis. Int J STD
2. Kuller LH, Tracy R, Belloso W, et al.: Inflammatory and coagulation
PubMed Abstract | Publisher Full Text
4. Triant VA, Meigs JB, Grinspoon SK: Association of C-reactive protein and HIV
in Malawian Adults Is Associated With the Proportion of PD-1-Expressing
CD8+ T Cells and Reverses With Antiretroviral Therapy. J Infect Dis. 2019;
for medical inpatients at Queen Elizabeth Central Hospital in Blantyre,
7. DART Trial Team; Mugerwa P, Walker AS, et al.: Routine versus clinically
driven laboratory monitoring of HIV antiretroviral therapy in Africa
PubMed Abstract | Publisher Full Text | Free Full Text
8. Heikinheimo T, Chimbayo D, Kumwenda J, et al.: Stroke outcomes in Malawi,
a country with high prevalence of HIV: a prospective follow-up study. PLoS
hypertension, and stroke in Malawian adults: A case-control study.
Publisher Full Text | Free Full Text


25. Malawi Liverpool Wellcome Clinical Research Programme: Circulating Microparticles are increased amongst people presenting with HIV and advanced immune suppression in Malawi and correlate closely with arterial stiffness. Ann Arbor, MI: Inter-university Consortium for Political and Social Research [distributor], 2021-08-18.


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This current paper by Kelly et al. is based on a study which was primarily aimed at characterising circulating microparticles in HIV-infected individuals. It was also aimed at assessing the association between CMPs with risk to developing cardiovascular “complications”? “diseases”?

The manuscript is well laid out and presents the results in an orderly manner. However, I have the following observations to make:

The manuscript needs to be re-written as there are quite a number of typos and some hanging statements (more details provided under Minor comments).

**Major comments:**

1. The authors point out under Methods/Study Cohort that “full details of recruitment and methods are available in Kelly et al., JID 2019”. However, the JID paper refers to a cohort of participants recruited into the REALITY study whereas this manuscript is based on the SHIELD study. The authors need to clearly explain if the two are one and the same. If not, how do the two studies differ?

2. Linked to point number one, the aim of the work in the JID paper was to characterize the contribution of immune activation to arterial stiffness in “HIV-infected Malawian adults initiating ART with advanced immunosuppression, compared with that in HIV-uninfected adults, and to determine how this changes over time on ART.”, suggesting that participants were recruited before they had initiated ART. In this paper the aim of the study is “to characterise CMPs in HIV[-infected] individuals (who are recruited at what stage?). It is important to state at what stage of the HIV infection the study participants are recruited. This is only mentioned in the Abstract but not in the main text.

3. The aim also states: "to assess their [CMPs?] relationship with cardiovascular risk and elucidate pathways that might be involved in "heightened" inflammation during early ART". The results however only provide:
3.1 association between absolute CMP counts, HIV-infection and arterial stiffness and
3.2 association between CMP subsets, HIV and arterial stiffness.

4. There are no sets of results showing the direct relationship between CMPs with risk to
develop cardiovascular complications. Therefore it would be better to rephrase the aim and
link it with what the results are showing. Similarly, no potential pathways possibly involved
in increased inflammation during early ART stages are provided and therefore this too
should be rephrased or removed.

5. In the Discussion section the authors need to include the possible limitations of the study.

**Minor comments:**

1. In the Abstract consider saying:

Background: “We investigated whether circulating microparticle (CMP) subsets were elevated in
individuals presenting with [untreated? ART-naïve? HIV] or HIV infection who had just commenced
ART”. The group of study participants needs to be clearly defined right from the start.
The Method section in the Abstract should read: “We characterized the microparticle and carotid
femoral pulse wave velocity of ART-naïve adults with a new HIV diagnosis and CD4 [count] <100
cells/μL two weeks post ART initiation”. Although the study cohort is characterized clearly in the
abstract, this detail is missing in the main text Methods section.

The last sentence in the Conclusion which starts: “Endothelial damage and platelet …” is rather
vague and should be re-written.

**Introduction:**

1. “but the underlying pathogenic mechanisms“ - of what specifically?

2. “15.6 among those within the first 6 months of ART...“ - please revisit the sentence and
consider re-writing it

3. “The increase in arterial stiffness previously identified ….”- please consider re-writing.

4. “Previous studies in [humans infected with?] HIV... " - please consider changing as
proposed.

5. Ethical considerations: “Ethical approval for the study was obtained from College of
Medicine Research and Ethics Committee (COMREC) of the University of Malawi
(P.09/13/1464) and from the University of Liverpool Research and Ethics Committee
(UoL000996). Written informed consent was obtained from each participant before taking
part in the study.

6. A [10-ml?] venous blood sample was collected in [EDTA tubes?] two weeks after ART
initiation. [After centrifuging?] plasma was extracted and stored [at -86°C?] until the day of
analysis.
7. Please consider revising the entire paragraph which starts “SHIELD participants [infected with HIV?] were ordered [arranged? Ranked?] ... “ - the entire paragraph does not make sense.

8. Under Statistics, please consider changing “...0.003 was used as significant” - it doesn't make sense.

9. Explain briefly how the agitation of the plates was done.

10. “350uL of each FACS tube was acquired” - please change to “350uL contents? Of each FACS tube was ...”.

11. Under Cell surface Immunophenotyping consider starting the sentence with “T cell subsets surface Immunophenotyping ...”.

Results:
1. What's the significance of the “education level of at least primary school completion”?

2. Please consider replacing “relationship” with “association”.

3. Please consider “Participants [infected?] with HIV”.

4. Table 1 - please consider presenting the Median results first on their own and have the Frequency results grouped together.

5. Figure 2- please consider including the rho values and the p value within the figure.

6. Figure 3 - it might be worthwhile including lines showing which two groups are being compared. E.g line between the dots for No HIV vs HIV.

Discussion:
1. The whole Discussion section sounds a bit “casually written” and could do with a bit of re-writing.

2. Specify that the immune suppression in the third line is HIV-infection related.

3. The sentence which starts “However, the association between CMPs and arterial stiffness was not reduced ... “ should be considered for revision as its not clear.

4. There is need for a reference at the end of the sentence “As well as acting as biomarkers of inflammation...pathogenesis”.

5. Limitations of the study to be included in the Discussion section.

References

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Partly

If applicable, is the statistical analysis and its interpretation appropriate? Yes

Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.