



RESEARCH ARTICLE

REVISED **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 2; peer review: 2 approved, 1 approved with reservations]**

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Abstract

Background: We aim to investigate whether circulating microparticle (CMPs) subsets were raised amongst people presenting with a new diagnosis of HIV and advanced immune suppression in Malawi, and whether they associated with arterial stiffness.

Methods: Microparticle characterisation and carotid femoral Pulse Wave Velocity (cfPWV) were carried out in a cohort of adults with a new HIV diagnosis and CD4 <100 cells/ μ L at 2 weeks post ART initiation. HIV uninfected controls were matched on age, systolic 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% CI 4 – 40%, $p = 0.02$).

In terms of subsets, endothelial and platelet derived microparticles

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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 PLWH in Malawi. Endothelial and platelet microparticles are the predominant cell origin types, indicating that platelet driven endothelial dysfunction pathways warrant further investigation in HIV associated arterial stiffness.

Keywords

HIV, microparticles, cardiovascular

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Any reports and responses or comments on the article can be found at the end of the article.



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REVISED Amendments from Version 1

The main changes to this version consist of clarifications in wording of text to make the analyses clearer for the reader.

Any further responses from the reviewers can be found at the end of the article

Introduction

People living with HIV (PLWH) are at increased risk of cardiovascular diseases such as myocardial infarction and stroke¹⁻⁴. Heightened inflammation has been implicated in vascular dysfunction but the underlying pathogenetic mechanisms have not been fully elucidated². 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 ART failure are prevalent⁵⁻⁷. In Malawi, several studies have shown an increased risk of stroke associated with HIV^{8,9}.

The first few months of antiretroviral treatment (ART) carry a particularly high cardiovascular risk. In a case control study of 705 patients, the odds of stroke were much higher within the first 6 months of ART compared to those who were treatment naïve (adjusted odds ratio 15.6), as well as in those with CD4 less than 200 cells/mm³ compared to those with CD4 greater than 500 cells/mm³ (adjusted odds ratio 12.9)⁹.

Arterial stiffness has been used as a measure of vascular dysfunction and cardiovascular risk in HIV studies as well as other chronic inflammatory conditions^{10,11}. cPWV is a gold standard measurement of arterial stiffness and has been validated as a physiological biomarker of cardiovascular events and mortality^{12,13}. Arterial stiffness is therefore used as a physiological marker of the risk of future cardiovascular events.

We have previously shown that amongst people presenting with HIV and a CD4 count less than 100 cells/mm³, arterial stiffness is increased by 12% (adjusted fold change) compared to healthy volunteers living without HIV⁵. However, this effect only persists during the first 3 months of ART and is associated with markers of inflammation, including T cell activation⁵. 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 VCAM, ICAM, and undergo transcytosis leading to the development of foam cells¹⁴. 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 cap¹⁵. During this process, elastin is degraded by enzymes such as MMPs and is replaced by collagen, increasing arterial stiffness¹⁵.

Circulating Microparticles (CMPs) are released into the circulation following inflammation driven activation or apoptosis

of the affected cells¹⁶. Through a process of blebbing, 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 surface¹⁷. 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 the field of 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 activation¹⁸⁻²¹. 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 HIV²². Here, we aim to take the characterisation of circulating microparticles in HIV a step further by assessing their relationship with cardiovascular risk, as measured by arterial stiffness, and by seeking to elucidate pathways that might be involved in heightened inflammation during early ART.

Methods**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 (in which some SHIELD patients were also co-recruited into the Reduction of EARly mortality in HIV-infected adults and children starting antiretroviral therapy (REALITY) clinical trial) has been reported on previously, and full details of recruitment are available in 5. In brief, ART-naïve adults with a new HIV diagnosis and CD4 <100 cells/μL underwent a detailed clinical assessment, blood draw for plasma storage and cPWV at 2 weeks post ART initiation. Study procedures were carried out a two weeks so as to avoid burdening the clinically vulnerable population at the initial ART visit. cPWV measured arterial stiffness using a Vicorder device (Skidmore Medical, London, UK) according to standardised guidelines²³. A random sample of wave forms was reviewed by an experienced independent assessor at three timepoints during the study to ensure consistent quality.

A convenience sample of 36 participants from the PLWH cohort was chosen in two groups. The first group were those with a cPWV in the highest quartile (>8.2 m/s), from which 24 participants were randomly chosen. The second were those with a cPWV in the bottom three quartiles (<8.2m/s), from which 12 participants were randomly chosen. The aim of having a 2:1 ratio between highest cPWV and lower cPWV values was 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 PLWH participants on age, systolic BP and diastolic BP in a 1:1 ratio.

Statistical analysis

Wilcoxon Ranksum and Spearman rho were used to test 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 the level for statistical significance. 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 previously⁵.

Laboratory procedures

Microparticle isolation. Plasma samples collected from 10ml sodium citrate tubes and 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 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 on a plate shaker. 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 1.1µm were used for microparticle gating, with microparticles 1µm 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 the contents of each FACS tube was acquired and the plots were then transferred to Flow Jo (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 previously⁵. The T-cell panel consisted of CD3 BV510, CD4 V450, CD38 PE Cy7, 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).

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.

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. 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 1.1×10⁵ copies/mL (0.4 – 2.6) respectively.

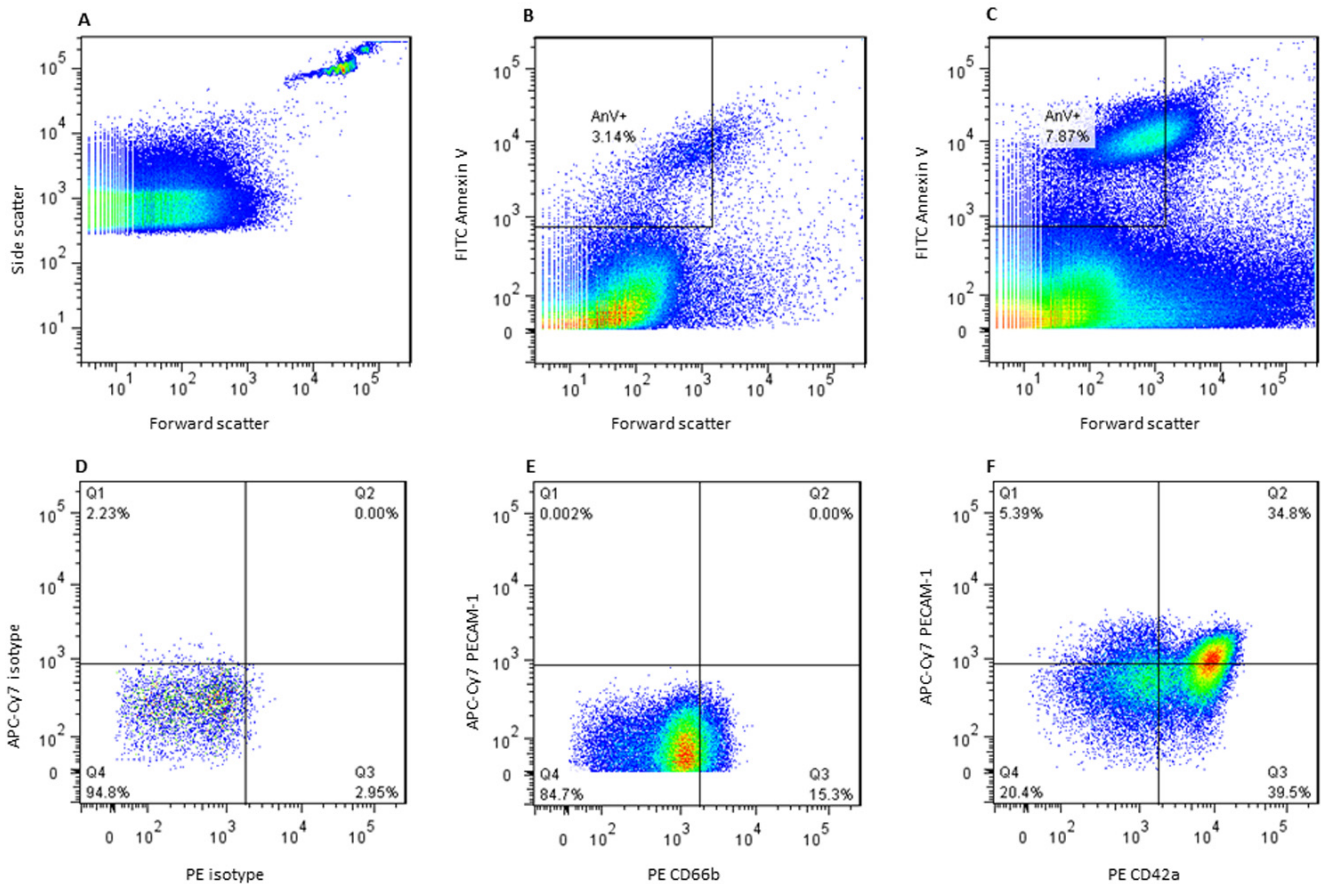


Figure 1. Identification of the microparticle population and subset gating strategy. **A.** 1.1 μ m beads were used to find forward scatter gate for microparticle identification **B.** Microparticle pellet was obtained from platelet poor plasma and stained for Annexin V. Total circulating microparticle count was measured by identifying microparticles Annexin V positive and less than 1.1 μ m 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.

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 PLWH compared to those without HIV (Table 1). Total CMP counts were also significantly associated with arterial stiffness (spearman rho 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 cfPWV [fold change 1.20m/s, 95% CI 1.04 – 1.40; $p = 0.02$]. When additionally adjusted for HIV status, the association between log total circulating microparticle count and cfPWV 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, 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 – 13.9)

Table 1. Clinical characteristics of 67 SHIELD participants with microparticle data according to HIV status.

	People living with HIV n=33		People living without HIV n=36	
	Median or Frequency	IQR or %	Median or Frequency	IQR or %
Age (years)	41	38 - 50	40	34 - 50
Female	13	39%	15	42%
Primary school education or less	16	48%	15	42%
Body Mass Index (kg/m ²)	21.7	19.4 - 24.7	22.1	19.4 - 23.8
Systolic BP (mmHg)	130	122 - 135	128	118 - 134
Diastolic BP (mmHg)	80	74 - 88	76	69 - 79
Haemoglobin (g/dL)	12	11 - 13	14	13 - 15
Cholesterol (mmol/L)	4.3	3.7 - 4.7	4.2	3.7 - 4.9
Glucose (mmol/L)	4.6	4.3 - 5.0	4.6	4.2 - 5.4
Creatinine (mmol/L)	68	58 - 83	66	57 - 80
History of ever smoking	6	18%	5	14%
History of a diagnosed cardiovascular disease	3	9%	4	11%
Co-infection at time of recruitment*	3	9%	1	3%
CD4 count (cells/ μ L)	42	31 - 71	-	-
HIV Viral Load ($\times 10^5$ copies/mL)	1.1	0.4 - 2.6	-	-
cfPWV (m/s)	9.1	8.2 - 10.3	8.0	6.7 - 8.7
Total Circulating Microparticle Count (log particle/mL)	6.7	6.3 - 7.3	5.6	5.3 - 5.8

*Acute co-infection, refers to diagnosis of an infection other than HIV.

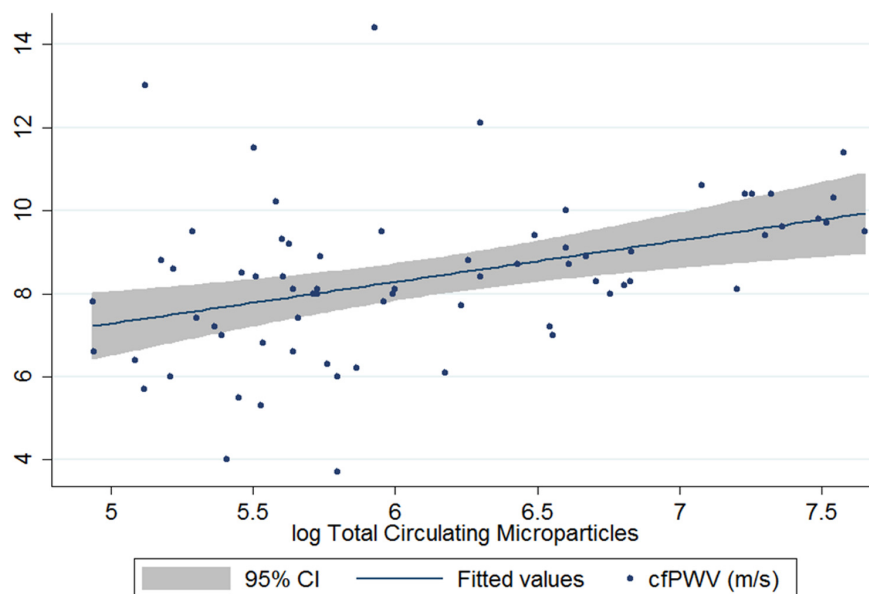


Figure 2. Association between total circulating microparticles and carotid femoral pulse wave velocity (m/s). Correlation analysis demonstrated spearman rho 0.47, $p < 0.001$.

Table 2. Linear regression analysis showing the association between total circulating microparticles and carotid femoral Pulse Wave Velocity. 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 cohort⁵. 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.

	Association between total circulating microparticle count and cfPWV			Association between total circulating microparticle count and cfPWV adjusting for HIV		
	Fold change	95% CI	P value	Fold change	95% CI	P value
Total Microparticle Count (per log particle/mL increase)	1.20	1.04 – 1.40	0.02	1.23	1.01 – 1.50	0.046
Age (per 10 year increase)	1.14	1.03 – 1.26	0.02	1.12	1.02 – 1.24	0.03
Female sex	0.85	0.67 – 1.07	0.18	0.84	0.66 – 1.07	0.17
Diastolic Blood pressure (per 10mmHg increase)	1.20	1.06 – 1.38	0.004	1.21	1.07 – 1.38	0.004
Haemoglobin (per g/dL increase)	1.04	0.98 – 1.10	0.19	1.03	0.96 – 1.10	0.41
HIV infection	-	-	-	0.95	0.66 – 1.35	0.76

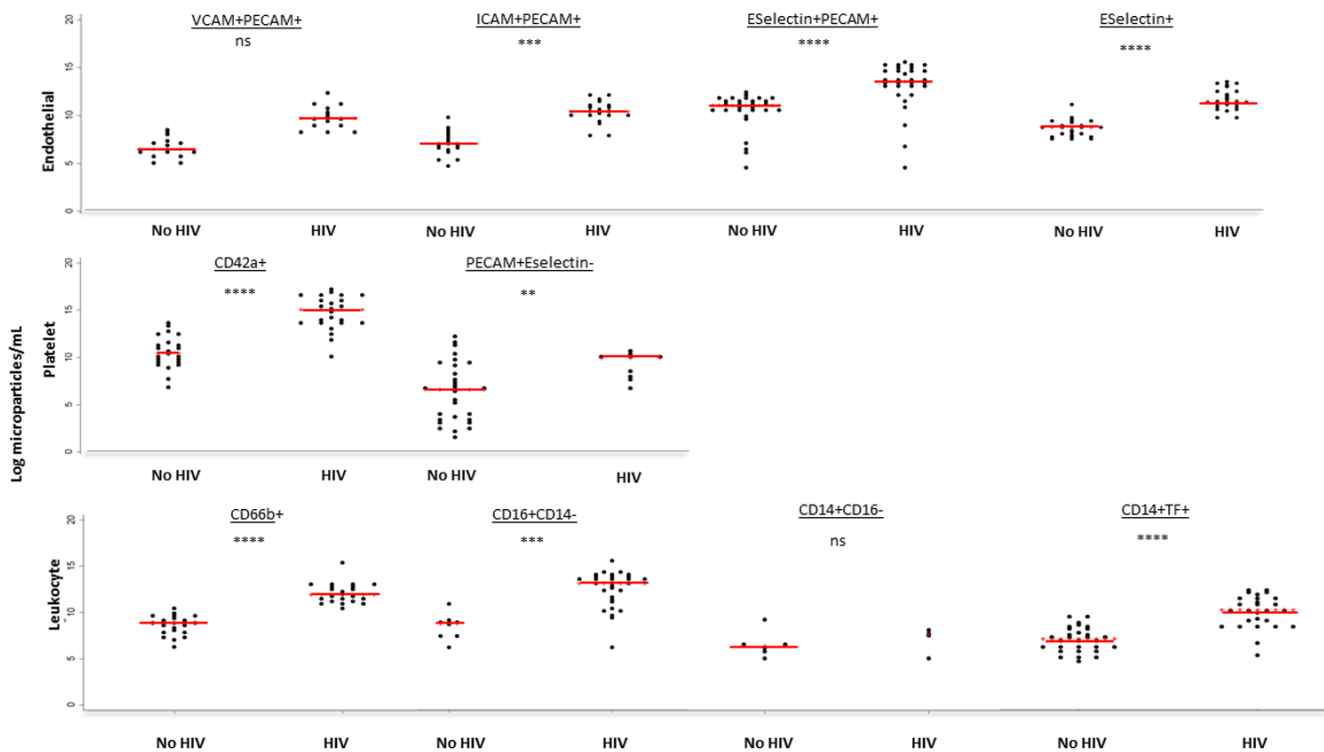


Figure 3. Microparticle subsets according to HIV Status for 69 Malawian Adults. PLWH=People living with HIV. No HIV= People living without HIV. Red lines represent median microparticles count. Grey Asterix represent p value: ns denotes p>0.003, * denotes p<0.003 but >0.0001, **denotes p<0.0001, ***denotes 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.

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 related 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 PLWH⁵. However, the association

between CMPs and arterial stiffness was not reduced when HIV was added as a variable to the model, 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 stress²⁴. 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 infection²², 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 presentation²⁵. 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 cytokines²⁶. CMPs have been shown to transfer important material such as miRNA and lipids to other cells²⁷. In particular, the transfer of the CCR5 receptor to endothelial cells from leucocyte derived microparticles potentially renders them permissible to direct HIV infection²⁸. Circulating microparticles produced in response to HIV could lead to endothelial damage, and therefore increased risk of cardiovascular disease, through multiple mechanisms including direct endothelial adhesion and activation, transfer of cytotoxic viral proteins, and propagation of low level viral replication.

We discovered that microparticles of endothelial and platelet origin are particularly expanded amongst PLWH in this low income SSA setting. Defining endothelial microparticles as CD51+, da Silva *et al.* previously reported endothelial microparticles 20 times higher in PLWH compared to people without HIV¹⁸. Endothelial microparticles promote expression of adhesion molecules on the endothelial cell surface, generating thrombosis, platelet activation and recruitment of inflammatory cells²¹. Platelet driven thrombosis in combination with endothelial activation may take advantage of a compromised endothelial barrier and lead to decreased elasticity and smooth muscle reactivity within the arterial wall²⁹. Inflammatory cytokine release in response to ongoing cellular recruitment, as well as from direct effects of the HIV virus, can also activate and increase the proliferation and migration of vascular smooth muscle cells, and thus induce arterial stiffness³⁰. Wheway *et al.* have previously found endothelial microparticles to form conjugates with T cell subsets, with increased binding to those cells that were pre-stimulated³¹. Binding was VCAM and ICAM dependent and endothelial microparticles were able to stimulate *in vitro* proliferation of T cells.

Table 3. Correlations between microparticle subsets and cfPWV.

Circulating Microparticle subset	Total		
	Spearman's rho	P value	
Endothelial	PECAM+CD42a-	0.63	<0.0001
	Eselectin+	0.61	<0.0001
	PECAM+Eselectin-	-0.34	0.004
	PECAM+Eselectin+	0.55	<0.0001
	PECAM-Eselectin+	0.53	<0.0001
	ICAM+PECAM+	0.33	0.02
	ICAM+PECAM-	0.58	<0.0001
	VCAM+PECAM+	0.26	0.07
	VCAM+PECAM-	0.46	<0.001
Leucocyte	CD66b+PECAM-	0.39	<0.01
	CD14-CD16+	0.74	<0.0001
	CD14+CD16+	0.08	0.78
	CD14+CD16-	-0.06	0.85
Platelet	CD42a+ PECAM-	0.61	<0.0001

Although this study is limited by a small sample size and its cross sectional design, we demonstrate quantification and characterisation of circulating 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 interesting 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. CMV, TB, 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.

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Reviewer Report 19 October 2022

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Wilson L. Mandala 

Malawi University of Science and Technology, Thyolo, Malawi

Having read the responses from the authors and the revised version of the manuscript, I would like to submit that I do not have any further comments to be made and I would recommend that this manuscript be unconditionally accepted for indexing without any further reservations from me.

Competing Interests: No competing interests were disclosed.

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.

Reviewer Report 17 October 2022

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Joseph Heskin 

Department of HIV, GUM, Chelsea and Westminster NHS Foundation Trust, London, UK

I have reviewed all responses to my original comments and am satisfied by the authors explanations or amendments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: HIV

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.

Version 1

Reviewer Report 30 August 2022

<https://doi.org/10.21956/wellcomeopenres.18825.r51610>

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**Siddharth Kogilwaimath**

¹ The Harvard T.H. Chan School of Public Health, Harvard University, Boston, MA, USA

² Division of Infectious Diseases, Department of Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Great work on this by Dr. Kelly and colleagues. Very interesting topic that is relevant in HIV medicine. I have left suggestions based on the headings:

Introduction:

- Well written overall.

Methods:

- Study Cohort: Unclear why the data was collected at 2 weeks into ART initiation. The lack of baseline data should be explained clearly. If IRIS is a plausible mechanism, could the choice of baseline ART used influence the total CMP expression? I am not sure if there was access to any INSTI based regimens during 2014-2015. I suspect that DTG was not widely used at that time but if an INSTI based regimen was used, is there a link with those on INSTI regimens and higher CMPs?
- Study Cohort: I am afraid it was not easy to follow the second paragraph. I would re-write this and simplify it.
- Laboratory procedures: Would suggest including a sentence on whether there is an established standard for CMP staining/gating and cell surface immunophenotyping and if so, how/why did this study standard differ?

Discussion:

- "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." especially around "when HIV was added"- I presume to the logistic regression model but this should be clarified.

- Would include what proportion of these PLWH had an active OI or malignancy related to advanced HIV as these could also affect CMPs I would imagine. I would at least include a comment on this in case data is unavailable or not collected and why? If data is available, I would consider including that.
- Consider including conclusion of an association between a biomarker and arterial stiffness based on data collected at a single time-point somewhere in the discussion. Was there longer term follow-up of the CMPs? Did it come down along with the arterial stiffness after 3 months on ART to suggest that it follows the same trajectory as the arterial stiffness based on prior studies? Did it come down as previously hypothesized to have been due to IRIS? Also, the sample size is extremely small and subject to confounding by indication with OIs/advanced HIV related malignancy.

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?

Yes

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: HIV, Infectious Diseases.

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.

Author Response 30 Sep 2022

Christine Kelly, UCD, Dublin, Ireland

Great work on this by Dr. Kelly and colleagues. Very interesting topic that is relevant in HIV medicine. I have left suggestions based on the headings:

Introduction:

- Well written overall.

Methods:

- Study Cohort: Unclear why the data was collected at 2 weeks into ART initiation. The lack of baseline data should be explained clearly. If IRIS is a plausible mechanism, could the choice of baseline ART used influence the total CMP expression? I am not sure if there was access to any INSTI based regimens during 2014-2015. I suspect that DTG was not widely used at that time but if an INSTI based regimen was used, is there a link with those on INSTI regimens and higher CMPs?

We thank the reviewer very much for the helpful comments. Study procedures were carried out to reduce initial burden on this clinically vulnerable population – for whom the priority was safely initiating ART. The ART regime at this time is laid out in the referenced JID paper (reference 5), but first line regimes at that time did not include DTG.

- Study Cohort: I am afraid it was not easy to follow the second paragraph. I would re-write this and simplify it.

This has been rewritten for clarity.

- Laboratory procedures: Would suggest including a sentence on whether there is an established standard for CMP staining/gating and cell surface immunophenotyping and if so, how/why did this study standard differ?

CMP is a research tool with no established standard for measurement as yet. The protocols contained within this paper were specifically developed by the authors.

Discussion:

- "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." especially around "when HIV was added"- I presume to the logistic regression model but this should be clarified.

This has been clarified accordingly and with thanks.

- Would include what proportion of these PLWH had an active OI or malignancy related to advanced HIV as these could also affect CMPs I would imagine. I would at least include a comment on this in case data is unavailable or not collected and why? If data is available, I would consider including that.

The numbers of patients with a concurrent infection at time of recruitment are shown in Table 1. The possibility that such infections may be contributing to inflammation over and above HIV is acknowledged within the limitations paragraph of the discussion section.

- Consider including conclusion of an association between a biomarker and arterial stiffness based on data collected at a single time-point somewhere in the discussion. Was there longer term follow-up of the CMPs? Did it come down along with the arterial stiffness after 3 months on ART to suggest that it follows the same trajectory as the arterial stiffness based on prior studies? Did it come down as previously hypothesized to have been due to IRIS? Also, the sample size is extremely small and subject to confounding by indication with OIs/advanced HIV related malignancy.

The small sample size and possible confounding by OIs have been highlighted as limitations in the penultimate paragraph of the discussion, which has now been flagged more clearly as a limitation section. We have added an additional limitation

around the cross sectional nature of the work.

Competing Interests: No competing interests were disclosed.

Reviewer Report 03 August 2022

<https://doi.org/10.21956/wellcomeopenres.18825.r51608>

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Joseph Heskin 

Department of HIV, GUM, Chelsea and Westminster NHS Foundation Trust, London, UK

This manuscript is well written and laid out. I note the following points which I feel can be easily resolved:

Major Comments

1. The cohort described in this paper are termed the SHIELD cohort and the paper reports that details of recruitment are in reference 5 (Kelly *et al.*, 2019). This paper refers to the REALITY trial and does not mention the SHIELD cohort. Please can the authors clarify if this is the same cohort.
2. Please can the authors explain why cfPWV measurements were not taken prior to the initiation of ART and only 2 weeks after initiation. This would appear to be important if you are using this as a marker of ART induced vascular dysfunction.

Minor Comments

Introduction:

1. Final paragraph "previous studies.." - please consider removing 'in HIV' as this is covered by discussion PLWH.

Methods:

1. Please consider switching the statements around so it reads that ethical approval was granted prior to participants providing informed written consent.
2. "SHIELD participants with HIV..." - it is unclear what is meant by ordered. Please consider rewriting the paragraph as it is not clear how and why convenience samples were chosen.
3. Were participants matched by gender?

Results:

1. What is the relevance of the participants attained educational level?

Discussion:

1. It is not clear from the recruitment whether participants living with HIV had active infections at the time of recruitment. Given the immunocompromised state, is there a concern for active OI's contributing to platelet and endothelial dysfunction?

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: HIV

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.

Author Response 30 Sep 2022

Christine Kelly, UCD, Dublin, Ireland

This manuscript is well written and laid out. I note the following points which I feel can be easily resolved:

Major Comments

1. The cohort described in this paper are termed the SHIELD cohort and the paper reports that details of recruitment are in reference 5 (Kelly et al., 2019). This paper refers to the REALITY trial and does not mention the SHIELD cohort. Please can the authors clarify if this is the same cohort.

We thank the reviewer for highlighting this point of confusion. A proportion of SHIELD participants were co-recruited to the REALITY trial, the details of which are outlined in the referenced paper. We did not specifically refer to the REALITY trial here as it was

not relevant to the current analysis. However, we have added a sentence to the methods to make it clear that the participants were co-recruited.

2. Please can the authors explain why cfPWV measurements were not taken prior to the initiation of ART and only 2 weeks after initiation. This would appear to be important if you are using this as a marker of ART induced vascular dysfunction.

Study procedures were carried out at 2 weeks post ART initiation because this was a clinically vulnerable and immunosuppressed group of patients. We therefore did not want to burden them with study procedures at the first ART visit in order to ensure understanding of clinical concepts.

A sentence clarifying this has been added to the manuscript.

Minor Comments

Introduction:

1. Final paragraph "previous studies.." - please consider removing 'in HIV' as this is covered by discussion PLWH.

We have added 'in the field of' to show highlight that CMPs have been studied elsewhere if the reader would like to consider these further.

Methods:

1. Please consider switching the statements around so it reads that ethical approval was granted prior to participants providing informed written consent.

This has been amended accordingly and with thanks.

2. "SHIELD participants with HIV..." - it is unclear what is meant by ordered. Please consider rewriting the paragraph as it is not clear how and why convenience samples were chosen.

This paragraph has been adjusted to make the selection process clearer for the reader.

1. Were participants matched by gender?

Gender was not found to be significantly associated with arterial stiffness in previous analyses of this cohort and therefore was not used as a variable for matching.

Results:

1. What is the relevance of the participants attained educational level?

Education level was included as an indicator of socio-economic status which has been shown to be associated with inflammation in PLWH in low-income settings.

Discussion:

1. It is not clear from the recruitment whether participants living with HIV had active infections at the time of recruitment. Given the immunocompromised state, is there a concern for active OI's contributing to platelet and endothelial dysfunction?

The numbers of patients with a concurrent infection at time of recruitment are shown in Table 1. The possibility that such infections may be contributing to inflammation over and above HIV is acknowledged within the limitations paragraph of the discussion section.

Competing Interests: No competing interests were disclosed.

Reviewer Report 26 October 2021

<https://doi.org/10.21956/wellcomeopenres.18825.r46515>

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Wilson L. Mandala 

Malawi University of Science and Technology, Thyolo, Malawi

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/uL 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

1. Kelly C, Mwandumba H, Heyderman R, Jambo K, et al.: HIV-Related Arterial Stiffness in Malawian Adults Is Associated With the Proportion of PD-1-Expressing CD8+ T Cells and Reverses With Antiretroviral Therapy. *The Journal of Infectious Diseases*. 2019; **219** (12): 1948-1958 [Publisher Full Text](#)

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.

Author Response 30 Sep 2022

Christine Kelly, UCD, Dublin, Ireland

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"?

We would think to thank the reviewer for their thoughtful and thorough comments. A sentence has been added to the introduction to provide clarity around the fact that arterial stiffness is a physiological marker of cardiovascular disease, closely correlated with future cardiovascular events (as evidenced in the referenced articles).

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).

We thank the reviewer for the suggestion and have amended throughout.

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?

The details of co-recruitment between SHIELD and REALITY are detailed in the

referenced 5, and although the REALITY aspect of the recruitment is not relevant to the analyses presented in this current paper, we appreciate where confusion may arise and have added a sentence to the methods to help clarify this.

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.

This point has been clarified in the main text to match the abstract.

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.

We have added a clarification to the aims as stated in the final sentence of the introduction to show that arterial stiffness is measuring cardiovascular risk. CMP subsets demonstrate different pathways that may be involved in inflammation by characterising the origins of microparticles, which are produced in response to stress and inflammation. This process is referred to in the first sentences of the fourth paragraph of the introduction.

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.

Throughout the manuscript we refer to cardiovascular risk, which is measured by arterial stiffness as a physiological marker for cardiovascular events. Much like we would use risk scores, such as the Framingham risk score to measure risk of cardiovascular events. Therefore, when we refer to cardiovascular risk, this is being measured by arterial stiffness. The relationship between arterial stiffness and cardiovascular events is explored in more detail within the referenced articles. We have added clarifications to the introduction and methods to explain this more clearly.

Inflammatory pathways were assessed by characterised microparticle subsets. The most important subsets were found to be of endothelial and platelet origin, as highlighted in the discussion.

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

The limitations are described in the penultimate paragraph of the discussion. They include:

- **Small convenience sample**

- **Cohort living with advanced HIV and risk of unmeasured confounders**
- **Limited generalisability**

We have added clarification to the start of the paragraph to flag more clearly that it deals with study limitations.

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/uL 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.

We have rephrased the abstract to make sure the population is clear from the start and that the methods section reads more clearly. We have maintained the passive voice for consistency throughout.

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

This sentence has been rewritten accordingly.

Introduction:

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

The underlying pathogenic mechanisms refers to the relationship between heightened inflammation and vascular dysfunction in people living with HIV.

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

This sentence has been rewritten accordingly and with thanks.

3. "The increase in arterial stiffness previously identified" - please consider re-writing.

This sentence has been rewritten accordingly and with thanks.

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

This sentence has been clarified; reference to these cohorts being people living with HIV (PLWH) is provided later in the sentence.

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.

This section has been added.

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 -86oC?] until the day of analysis.

These details have been outlined within the microparticle isolation section.

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.

Clarifications have been made to this paragraph which we hope will make it clearer for the reader.

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

This sentence has been added to for clarity.

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

This has been added to the methods.

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

This has been changed accordingly.

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

Both T cell subsets and monocyte subsets were assessed as detailed in the remainder of the sentence.

Results:

1. What's the significance of the "education level of at least primary school completion"?

Education level gives an indication of the socio-economic status of the participants. Socio-economic status may be associated with risk of inflammation.

2. Please consider replacing "relationship" with "association".

We like the word relationship to describe this analysis.

3. Please consider "Participants [infected?] with HIV".

This has been changed to PLWH as standard terminology.

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

We feel that making two separate tables for characteristics may affect the fluidity of the results section for the reader.

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

We have added the rho and p values to the figure legend.

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.

Due to the large number of graphs in this figure it is difficult to add lines without making the figure over crowded.

Discussion:

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

We are happy with the tone of the discussion, aimed for the general reader. We have addressed the reviewer's specific queries regarding the discussion section below.

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

The word related has been inserted for clarity.

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.

This sentence has been clarified.

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

The references for the different mechanisms in which microparticles have been implicated in inflammation appear in the subsequent sentences that detail the individual mechanisms.

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

The penultimate paragraph deals with limitations as outlined above, and has now been flagged more clearly as a limitations section.

Competing Interests: No competing interests were disclosed.
