

Multimodal Circulating Myeloid Cell Analysis in Peripheral Artery Disease Reveals Neutrophil Priming and Monocyte Dysfunction

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Abstract

Peripheral artery disease (PAD) is a common age-associated cardiovascular disease, but how circulating innate immune cells are remodelled in PAD remains incompletely understood. Here, we performed an integrated analysis of circulating myeloid cells in PAD patients with intermittent claudication and healthy controls, combining high dimensional flow-cytometric immunophenotyping, *ex vivo* functional assays, plasma inflammatory markers, transcriptomic profiling of neutrophils and monocytes, and exploratory epigenetic analysis of neutrophils. PAD was associated with expansion of circulating neutrophils, conventional dendritic cells, and monocyte subsets, together with altered CD162⁺ myeloid cell profiles. Several of these differences persisted after adjustment for age, body mass index, and smoking status. Functionally, PAD neutrophils showed enhanced IL-6 and IL-8 production after LPS stimulation but reduced phagocytic capacity and lower intracellular reactive oxygen species. PAD was also associated with elevated circulating myeloperoxidase, without increased circulating NET-associated markers. In contrast, monocytes displayed attenuated cytokine production and impaired phagocytosis despite increased absolute counts. Transcriptomic analysis revealed an enrichment of inflammatory pathways, including TLR/NLR/NF- κ B and JAK–STAT signalling, and reduced enrichment of mitochondrial oxidative phosphorylation and autophagy programs in PAD neutrophils, whereas classical monocytes showed no significant resting-state differential gene expression. These findings identify systemic myeloid immune remodelling as a feature of PAD and suggest cell type-specific innate immune dysfunction in the context of aging.

Introduction

Peripheral artery disease (PAD) is a common age associated cardiovascular disorder characterised by atherosclerotic obstruction of the lower-extremity arteries. Globally, PAD affects more than 200 million people, and its prevalence increases markedly with age, affecting up to one in five individuals aged ≥ 60 years^{1–3}. PAD is associated with impaired mobility, reduced quality of life, increased cardiovascular risk, and reduced health span. PAD presents a broad clinical spectrum, ranging from asymptomatic disease to intermittent claudication (IC) and chronic limb-threatening ischemia (CLTI). As a manifestation of advanced systemic atherosclerosis, PAD carries substantial morbidity, mortality, and economic burden, yet remains underrecognized and undertreated compared with coronary artery disease (CAD)^{1–3}.

Aging is accompanied by chronic low-grade inflammation and remodelling of innate immunity, processes that may amplify vascular inflammation, impair tissue repair, and contribute to atherosclerotic progression⁴. Myeloid cells, including neutrophils and monocyte subsets, are central mediators of inflammatory and reparative responses and are increasingly recognized as active contributors to cardiovascular disease⁵. Endothelial activation promotes leukocyte adhesion and transmigration into the arterial intima, representing an early immuno-inflammatory step in atherogenesis. Monocytes are recruited through chemokine and adhesion-molecule pathways, including CCL2–CCR2 and VCAM-1/ICAM-1, and differentiate into macrophages and dendritic cells that regulate lipid uptake, efferocytosis,

cytokine signalling, and plaque composition⁶. Circulating monocyte subsets: classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺), and non-classical (CD14⁺CD16⁺⁺), associate differentially with atherosclerotic burden and have been reported as independent predictors of future cardiovascular events and their outcome after myocardial infarction^{7,8}. Within plaques, macrophage polarization and defective efferocytosis promote necrotic core growth and fibrous-cap weakening, mechanisms linked to limb ischemia and thrombosis⁶.

Neutrophils, the most abundant type of leukocyte in human blood and key effectors of acute inflammation, were historically underappreciated in chronic vascular disease but are now recognized as active drivers of cardiovascular inflammation⁹. Through phagocytosis, degranulation, myeloperoxidase (MPO) activity, reactive oxygen species (ROS) generation, and formation of neutrophil extracellular traps (NETs), they destabilize endothelium, oxidize lipoproteins, and propagate inflammation^{9,10}. Clinically, higher circulating neutrophil counts are associated with adverse cardiovascular events in patients with acute coronary syndromes and PAD^{11,12}. Moreover, neutrophil-to-lymphocyte ratio (NLR) has emerged as a marker of systemic inflammation and atherosclerosis burden and has been reported as predictor of disease severity in PAD patients^{13,14}.

Crosstalk between neutrophils and monocytes may further intensify arterial inflammation. NET-associated components can enhance monocyte recruitment and influence macrophage polarization, whereas monocyte-derived cytokines and tissue factor can promote neutrophil activation and immunothrombosis^{15,16}. MPO, produced predominantly by neutrophils and to a lesser extent by monocytes and macrophages, has been implicated in CAD, plaque vulnerability, and plaque rupture¹⁷. Despite these links, relatively little is known about the circulating myeloid compartment in PAD, particularly with respect to the immunophenotype, intrinsic transcriptional programs, epigenetic features, and functional capacity of neutrophils and monocyte subsets.

Whether PAD is associated with disease-specific reprogramming of circulating myeloid cells beyond the effects of chronological aging remains unclear. We hypothesized that PAD is characterised by coordinated remodelling of circulating neutrophil and monocyte compartments toward altered inflammatory and effector states that associate with disease severity. To test this, we integrated deep immunophenotyping of circulating neutrophils and monocyte subsets with functional assays assessing phagocytosis, cytokine production capacity, MPO, ROS production, and circulating NET-associated markers. We further performed RNA sequencing of sorted neutrophils and classical monocytes, exploratory DNA methylation analysis of circulating neutrophils, and examined associations with clinical vascular parameters, including ankle–brachial index (ABI), carotid intima–media thickness (IMT), and carotid vessel diameter.

Material and Methods

Study population

Participants were recruited between October 2021 and March 2024 from the PIMM (PAD inflammation, microbiome and metabolites) study, a case–control study including symptomatic PAD patients with intermittent claudication and healthy controls (HCs). Patients with PAD were recruited from the Department of Cardio-thoracic and Vascular Surgery, University Hospital Örebro (USÖ), Sweden, whereas HCs were recruited at the School of Medical Sciences, Örebro University, Sweden. Participants were not individually age- or sex-matched. Because patients with PAD were older than HCs, age was included as a covariate in adjusted analyses where appropriate; sex distribution was comparable between groups and was therefore not included as an adjustment variable.

Exclusion criteria included dementia, autoimmune disease, inflammatory bowel disease, irritable bowel syndrome, kidney disease, diabetes, other inflammatory conditions, active infection, or use of antibiotics, probiotics, or bowel-cleansing preparations within 2 months before study entry. Clinical data, including disease duration, medication use, and comorbidities, were extracted from participants' medical records. Demographic characteristics and smoking status were obtained through self-report questionnaires.

Written informed consent was obtained from all participants prior to sample collection, in accordance with the Declaration of Helsinki. Ethical approval for this study was granted by the Swedish Ethical Review Authority (Dnr 2021–03386).

Physiological assessment and evaluation of quality of life

Participants underwent non-invasive examinations to assess cardiovascular risk by evaluating microvascular status, vessel diameter and IMT. The ABI and Toe-brachial index (TBI) were measured using a pen-Doppler and a laser-Doppler system (Perimed AB, Sweden) respectively to assess microvascular status in the limbs. In addition, IMT of the vessels was evaluated by ultrasound of the carotid arteries, performed at the Physiology Clinic, University Hospital Örebro. Participants also completed the VascuQoL6 health-state questionnaire to assess their quality of life. This questionnaire is routinely used in clinical practice and is included in the Swedish national quality register, SWEDVASC¹⁸.

Whole blood collection

Venous whole blood was collected from each participant after a minimum of 12 hours fasting period. The samples were drawn into sodium heparin, Ethylenediaminetetraacetic acid (EDTA), lithium heparin tubes, and Cell prep tubes (CPT) containing sodium heparin and ficoll (Becton Dickinson (BD) Biosciences, Franklin Lakes, NJ, USA). Blood samples collected in EDTA tubes were subsequently centrifuged, thereafter plasma was aliquoted and stored at -80°C until further analyses. Sodium heparin blood was used immediately for immunophenotyping and functional analysis by flow cytometry. Lithium heparin and EDTA tubes were analysed at the clinical chemistry laboratory at University Hospital Örebro for traditional cardiovascular risk marker analysis.

Total white blood cells (WBCs), neutrophil, and lymphocyte counts were measured in EDTA blood from each participant by using an automated haematology analyser (Swelab alfa plus, Sweden). The obtained WBCs count was used to calculate absolute cell counts (cell count/L) and neutrophil and lymphocyte counts were used to calculate NLR.

Peripheral blood mononuclear cell isolation

CPT tubes were centrifuged within two hours of blood collection, according to the manufacturers protocol. Peripheral blood mononuclear cells (PBMCs) were harvested and washed twice in phosphate-buffered saline (PBS, Gibco Thermo Fisher Scientific, Waltham, MA, USA) and resuspended in complete medium (RPMI 1640 medium supplemented with 10% Fetal bovine serum (FBS), PEST (100U/ml penicillin, 100µg/ml streptomycin), 5 µM 2-mercaptoetanol, 10 mM Hepes, 2 mM L-glutamin, Thermo Fisher Scientific). Live cells were counted by trypan blue (Sigma-Aldrich, MO, USA) using Bürker Chamber and 8–10 × 10⁶ cells were allocated for immediate monocyte staining for cell sorting. Residual cells were used to perform different functional analyses.

Flow cytometry

Samples were acquired using a Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with four lasers. Immune cells were sorted using Moflo Astrios cell sorter (Beckman Coulter). Data were analysed with Kaluza analysis software, version 2.3 (Beckman Coulter).

Surface staining

The phenotype of whole blood was analysed for the expression of neutrophil, conventional dendritic cells (cDCs), and monocyte specific cell surface markers by high dimensional flow cytometry. Whole blood was incubated with fixable viability staining (FVS) 510 (BD Biosciences) followed by incubation with Fc receptor blocking (FcR-block, BD Biosciences) for respectively 10 minutes. Without washing, monoclonal fluorescent antibodies (CD45, HLA-DR, CD14, CD16, CD66b, CD11c, CD162 and antibodies against lineage markers to exclude CD3, CD19, CD20, CD56 positive cells, Supplementary table 1) were added and incubated for 10 minutes. Subsequently, whole blood was lysed for 10 minutes using 1× FACS™ lysing solution (BD Biosciences). Intracellular MPO staining was performed for 30 minutes after fixation and permeabilization (1× FACS Permeabilizing Solution 2, BD Biosciences) and finally resuspended in FACS buffer (PBS supplemented with 2% heat-inactivated FBS and 2 mM EDTA) for flow cytometry analysis. The proportions of neutrophils (CD66b⁺CD16⁺), cDCs (CD16⁻CD14⁻CD11c⁺), and monocyte subsets (classical: CD14⁺⁺CD16⁻, intermediate: CD14⁺⁺CD16⁺ and non-classical: CD14⁺CD16⁺⁺) were assessed. Expression of MPO and CD162 (P-selectin glycoprotein ligand-1, PSGL-1) was subsequently analysed on neutrophils and each monocyte subsets. Fluorescence minus one (FMO) and unstained controls were used to set the gates (gating strategy; Supplementary Fig. 1A).

Phagocytosis assay and Intracellular reactive oxygen species (ROS)

To assess the functional characters of neutrophils and monocytes we performed phagocytosis assay and quantified intracellular ROS levels. Uptake of pHrodo™ *E. coli* BioParticles® were used to quantify phagocytosis in whole blood cells using flow cytometry. Whole blood samples were treated either with cytochalasin D (10 µg/ml, inhibitory control, Sigma-Aldrich, St. Louis, MO, USA) or without (positive control) for 30 minutes 37°C in 5% CO₂. Following pre-treatment, pHrodo™ *E. coli* BioParticles® (0.2 mg/ml, Sigma-Aldrich) were added, and samples were incubated for 1 hour under the same conditions. Samples were put on ice for 5 minutes, followed by incubation with monoclonal fluorescent antibodies (CD45, HLA-DR, CD14, CD16, CD66b and lineage of antibodies to exclude CD3, CD19, CD20, CD56 positive cells, Supplementary table 1) for 10 minutes. Erythrocytes were then lysed (1x FACS™ lysing solution, BD Biosciences) for 10 minutes, and the cells were washed and further resuspended in FACS buffer for flow cytometry analysis. Median fluorescence intensity (MFI) of neutrophils and monocyte subsets (classical monocytes and non-classical monocytes) expressing pHrodo were assessed. Percentage inhibition of phagocytosis was calculated by subtracting the inhibitory control from the positive control and dividing the results by the positive control. Gating strategy is described in Supplementary Fig. 1B.

Intracellular ROS was determined using 2,7-Dichlorodihydrofluorescein diacetate (H2-DCFDA, Thermo fisher Scientific). Whole blood was incubated with H2-DCFDA (10 µM) in dark for 30 min at RT, followed by 1 hour stimulation with and without phorbol 12-myristate 13-acetate (PMA, 100 nM, Sigma-Aldrich). Whole blood was stained with monoclonal fluorescent antibodies (CD14, CD16, and CD66b, Supplementary table 1) for 15 minutes and subsequently incubated for 10 minutes with 1x FACS™ lyse (BD Biosciences). After centrifugation, the samples were washed and resuspended in FACS buffer for flow cytometry analysis. MFI of stimulated cells for each sample was normalized against the unstimulated control. Gating strategy is described in Supplementary Fig. 1C.

Intracellular cytokine/chemokine staining

To assess cytokine and chemokine production, intracellular staining was performed on neutrophils and monocyte subsets following lipopolysaccharide (LPS) or PMA stimulation. Whole blood was diluted 1:1 with complete medium. Diluted blood was stimulated for 4 hours 37°C in 5% CO₂ with Brefeldin A (BFA, 1.0 µg/mL, BD Biosciences) and monensin (2.0 µM, BD Biosciences) or BFA, monensin and PMA (10 ng/ml, Sigma-Aldrich) or BFA, monensin and LPS (1 ng/ml, Sigma-Aldrich). After 4 hours incubation, whole blood was lysed for 10 minutes with 1x Pharm Lyse™ (BD Biosciences) and washed with FACS buffer. Cells were then incubated with FVS 520, followed by FcR-block (BD Biosciences) for respectively 10 minutes. Samples were then stained for surface monoclonal fluorescent antibodies (HLA-DR, CD14, CD16, CD66b and lineage of antibodies to exclude CD3, CD19, CD20, CD56 positive cells, Supplementary table 1) for 15 minutes. After further washing with FACS buffer, samples were incubated in fixation/permeabilization buffer (BD Biosciences) for 20 minutes, followed by 10 minutes incubation with

permeabilization/wash buffer. Finally, cells were stained for intracellular cytokines and chemokine (Interleukin (IL)-6, tumor necrosis factor-alpha (TNF- α), IL-1 β , and IL-8, Supplementary table 1) for 30 minutes, followed by washing with permeabilization buffer and resuspended with FACS buffer for following flow cytometry analysis. Fluorescence minus one (FMO), unstained controls and unstimulated controls (with only BFA and monensin) were used to set the gates (gating strategy; Supplementary Fig. 1D). MFI of stimulated cells for each sample was normalized against the unstimulated control.

PBMC stimulation

The functional profile of PBMCs was assessed using a 3D cell culture system with nanofiber scaffolds (Cellevate, Lund, Sweden). PBMCs were cultured in cRPMI-10 (RPMI 1640 medium supplemented with 10% FBS) in the presence or absence of LPS (1 ng/ml, Sigma-Aldrich) for 48 hours at 37°C in 5% CO₂. After incubation, culture supernatants were collected, and the levels of inflammatory markers IL-1 β and TNF- α were measured by ELISA according to manufacturer's instructions (R&D systems, USA).

Quantification of pro-inflammatory mediators in plasma

Soluble proteins were measured with 3 plex (IL-1 β , IL-6, IL-8) using human pro-inflammatory V-Plex kit (Meso Scale Discovery, Rockville, MD) detected by electrochemiluminescence in Meso QuickPlex SQ 120 (Meso Scale Discovery), according to the manufacturer's instructions. Samples were analysed using Meso Scale Discovery workbench analysis software. Levels of different pro-inflammatory mediators were expressed in pg/mL derived to a 5-parameter logistic standard curve for each cytokine. All samples were randomized and analysed in duplicate.

Soluble Myeloperoxidase and Neutrophil extracellular traps components

Soluble MPO levels in plasma were quantified using a commercially available MPO ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions. NETs components were analysed in plasma using the citrullinated histone H3 (CitH3; Clone 11D3) ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) and MPO-DNA complexes measured using a modified Cell Death Detection ELISA kit (Roche Diagnostics, Nederland B.V., Almere, The Netherlands), with an anti-MPO monoclonal antibody (clone 4A4, ABD Serotec) used as capture antibody, following the respective protocols. Absorbance was measured with a microplate reader (Epoch 2, Biotek, Winooski, VT, USA). Soluble MPO levels were expressed in μ g/L, CitH3 levels in ng/mL, and MPO-DNA levels in optical density (450/540). All samples were randomized and analysed in duplicate. Control samples with known MPO and MPO-DNA concentrations (low and high) were included in each ELISA run.

Cell sorting of neutrophils and monocytes subsets

Whole blood was incubated with FVS 510, followed by with FcR- block (BD Biosciences) for respectively 10 minutes. Subsequently, monoclonal fluorescent antibodies (CD45, CD66b, and CD16, Supplementary table 1) were added and incubated for 10 minutes. Whole blood was lysed for 10 minutes using 1x

Pharm lyse (BD Biosciences), followed by centrifugation. Cells were then washed once with FACS buffer and resuspended in FACS buffer for subsequent cell sorting of CD66b⁺CD16⁺ neutrophils (gating strategy; Supplementary Fig. 2A).

For sorting of monocyte subsets, PBMCs were incubated with live/dead staining FVS 570, followed by incubation with FcR- block (BD Biosciences) for respectively 10 minutes. Cells were then stained with monoclonal fluorescent antibodies cocktail (HLA-DR, CD45, CD14, CD16 and antibodies against lineage markers to exclude CD3, CD19, CD20, CD56 positive cells, Supplementary table 1) for 20 minutes at 4 °C, washed once with FACS buffer and resuspended in FACS buffer for cell sorting of monocyte subsets (classical: CD14⁺⁺CD16⁻, intermediate: CD14⁺⁺CD16⁺, and non-classical: CD14⁺CD16⁺⁺) (gating strategy; Supplementary Fig. 2B).

Sorted cells were collected into sterile FACS tubes containing complete medium, with at least 200,000 cells obtained per sample. Post-sort purity exceeded 98% for all cell populations. Sorted neutrophils were lysed in either RLT buffer containing β -mercaptoethanol or ATL buffer and stored at - 80°C for subsequent RNA-sequencing and epigenetic analyses. Monocyte subsets were processed identically; however, due to insufficient yields of intermediate and non-classical monocytes during sorting, only classical monocytes were included in the RNA-sequencing for mRNA expression analysis.

RNA-sequencing

Total RNA from lysates from sorted neutrophils and classical monocytes (HCs (n = 20; PAD, n = 20) was extracted with a RNeasy® PLUS Micro kit (Qiagen) according to the manufacturer's protocol.

Total RNA was subjected to quality control using Agilent Tapestation according to the manufacturer's instructions. Monocyte libraries were prepared with the Illumina TruSeq Stranded mRNA ligation protocol, including cDNA synthesis, adapter ligation, and amplification of indexed libraries, while neutrophil libraries were generated using the SMART-Seq mRNA LP Kit (TaKaRa Bio USA, Mountain View, CA, USA). For the latter, first-strand cDNA was synthesized using non-templated nucleotides added by SMARTScribe Reverse Transcriptase, followed by double-strand synthesis through template switching, after which indexed libraries were prepared by enzymatic fragmentation, ligation of stem-loop adapters, and nick extension. The yield and quality of libraries were assessed using Qubit (Thermo Fisher) and Agilent Tapestation. All libraries were purified, normalized, and pooled before sequencing. Monocyte libraries were sequenced on the Illumina NextSeq 1x NovaSeq XPlus 10B-300 lane, and neutrophil libraries on an Illumina NovaSeq 25B flowcell, both generating 2 × 150 bp paired-end reads. Basecalling and demultiplexing were performed using BCL2 software with default settings, producing Fastq files for downstream mapping and analysis.

For gene expression analysis, mRNA-seq reads were aligned to the human reference genome GRCh38, obtained from Ensembl, using the STAR aligner. Gene-level read counts were generated using featureCounts from the Subread package by assigning reads overlapping annotated exons to their corresponding genes based on Ensembl gene annotations. The resulting matrix of raw counts (genes ×

samples) was imported into R and we employed edgeR (Bioconductor) for differential expression analysis using the generalized linear model (GLM) pipeline, with the design matrix specifying the experimental conditions. Genes with low counts across samples were filtered out using edgeR's filterByExpr function to retain only those with sufficient expression for reliable inference. Library sizes were normalized using the trimmed mean of M-values (TMM) method to account for compositional differences between samples. Tagwise dispersions were used as an estimation of biological variability. GLMs were then fitted for each gene, and differential expression was assessed using contrasts between PAD patients and healthy controls within the GLM framework. Resulting p-values were adjusted for multiple testing using the Benjamini–Hochberg procedure to control the false discovery rate (FDR). Genes with $FDR < 0.05$ were considered significantly differentially expressed.

Gene set enrichment analysis (GSEA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

Gene set enrichment and pathway analyses were performed using the edgeR framework on the results of the differential expression analysis. For over-representation testing, Gene Ontology (GO) terms and KEGG pathways were evaluated with edgeR's implementation of goana and kegga, using all expressed genes that passed filtering as the background universe. Entrez gene identifiers (or Ensembl IDs mapped to Entrez IDs) were used for annotation. For each gene set, enrichment statistics were calculated based on the direction and magnitude of differential expression, and p-values were adjusted for multiple testing using the Benjamini–Hochberg procedure to control FDR. A cutoff p-value of 0.05 was used to filter the significant enrichment thereafter FDR with < 0.05 and < 0.1 for GO terms and KEGG pathways respectively were considered significantly enriched.

To perform gene set enrichment analysis (GSEA-type tests) using the full ranked gene lists, we applied the rotation-based methods available in the edgeR/limma framework (e.g. camera and/or romer) on the same negative binomial GLM fit used for differential expression. Curated gene sets were first converted to indices matching the row names of the expression object and tested competitively against the complement of genes in the dataset while accounting for inter-gene correlation within each set. For each contrast of interest, we obtained directional enrichment statistics and FDR-adjusted p-values and reported gene sets showing significant enrichment ($FDR < 0.05$) as biologically relevant pathways.

Methylation analysis

Total DNA was extracted from sorted neutrophils from 18 PAD and 16 HCs samples with QIAamp® DNA Micro kit (Qiagen) following the manufacturer's protocol. A total of 340 ng of high-quality genomic DNA was bisulphite-converted with EZ 96 DNA Methylation kit (Zymo Research, Irvine, CA, USA). DNA methylation analysis was subsequently performed using the Infinium MethylationEPIC Bead Chip v2.0 (Illumina, San Diego, CA, USA) in accordance with the manufacturer's protocol. Raw data were processed and analysed using the ChAMP pipeline (R package v2.21.1). Probes were excluded if they met any of the following criteria: a detection $p > 0.01$, localization on a single nucleotide polymorphism (SNP),

mapping to sex chromosomes (X or Y), a bead count < 3, or classification as a non CpG site. Samples were excluded if the missing probe rate exceeded 0.01 across CpG sites. Following quality control and filtering, data normalization using the BMQI method, after which differential methylation analysis was conducted on a final dataset comprising 12 PAD patient and 13 HC samples. Delta beta ($\Delta\beta$) value ≥ 0.15 was considered biologically relevant, given that sorted cells were used for analysis.

Statistical analysis

Statistical analyses and figure generation were performed using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA). RNA-sequencing and methylation analyses were performed using R packages. As most continuous variables were non-normally distributed, data are presented as medians unless otherwise stated. Comparisons between PAD patients and HCs were performed using the Mann–Whitney U test. Correlations between clinical parameters, ABI, carotid IMT, carotid vessel diameter, disease duration, CRP and immune markers in PAD patients were assessed using Spearman’s rank correlation coefficient. A two-sided p-value < 0.05 was considered statistically significant. Additional adjustment analyses were performed using IBM SPSS Statistics version 30.0 (IBM Corp., Armonk, NY, USA). For each immune marker, a univariate general linear model was used to assess differences between PAD patients and HCs after adjustment for age, BMI, and smoking status as potential confounders. Adjusted p-values for the group effect were extracted from the Tests of Between-Subjects Effects table for each marker.

Results

Patient population characteristics

Fifty HCs and 43 patients with PAD presenting with intermittent claudication were enrolled in a case–control study. For follow-up assays, randomly selected subsets of participants from each group were analysed. Compared with HCs, patients with PAD were older, had a higher body mass index (BMI), and more frequently had a history of hypertension and hyperlipidaemia (Table 1). Most patients with PAD were receiving antihypertensive and lipid-lowering therapy. Overall comorbidity burden was higher in the PAD group, with prior malignancy and previous vascular surgery also being more common than in HCs.

Clinical data revealed that macrovascular status was markedly impaired in PAD patients, as indicated by a significantly lower ABI and TBI compared to HCs (Table 2). The median ABI of 0.61 (min-max; 0.04–1.12) suggests that PAD patients in our study had mild to moderate claudication. Increased median vessel diameter of carotid arteries, as well as higher maximum and average carotid IMT was noted in PAD patients compared to HCs (Table 2). Furthermore, PAD patients reported a significantly lower VascuQoL6 score than HCs, suggesting a declined quality of life in PAD patients (Table 2).

Traditional cardiovascular risk markers, total cholesterol and low-density lipoprotein (LDL) cholesterol levels were significantly lower in PAD patients, reflecting greater statin use compared with HCs. High-density lipoprotein (HDL) cholesterol was also markedly reduced, whereas triglycerides were higher in

PAD patients (Table 2). The NLR was elevated in PAD (Table 2), indicating greater systemic inflammation. Creatinine did not differ between groups. Estimated glomerular filtration rate (eGFR) was similar among women; in men, eGFR was lower in PAD than in HCs (Table 2).

Table 1
Patient characteristics.

Demographics	HC n = 50	PAD n = 43	<i>p value</i>
Age, mean (SD)	72 (3.5)	77 (6.5)	0.0001
BMI (kg/m ²), median (min-max)	23.4 (20.1–29.5)	25.4 (19.7–35.4)	0.0043
Sex, M/F	23/27	25/18	
Clinical history n (%)			
Hypertension	4 (8)	29 (67)	
Hyperlipidaemia	1 (2)	20 (46)	
Active smoker	2 (4)	13 (31)	
Non- smoker	27 (54)	9 (21)	
Former smoker	21 (42)	20 (48)	
Comorbidities n (%)			
Previous vascular surgery	1 (2)	13 (30)	
Heart failure	0 (0)	8 (19)	
Ischemic heart disease	2 (4)	13 (30)	
Aortic valve disease	0 (0)	4 (10)	
Arrhythmias	1 (2)	9 (21)	
Malignancy history	5 (10)	15 (35)	
COPD	0 (0)	7 (16)	
CKD	0 (0)	2 (5)	
Medication past 12 months n (%)			
Hypertension medication	8 (16)	33 (77)	
Antiarrhythmics medication	5 (10)	26 (60)	
blood thinning medication	2 (4)	10 (23)	
Antiplatelet therapy	2 (4)	32 (74)	
Statins	7 (14)	34 (79)	

Demographics	HC	PAD	<i>p value</i>
	n = 50	n = 43	
Other lipid-lowering drugs	5 (10)	11 (26)	
Anti-inflammatory treatment/NSAIDs	5 (10)	4 (9)	
Immunosuppressive therapy	2 (4)	4 (9)	

HC; missing 1; BMI.

PAD; missing 1; smoking.

BMI, body mass index, M/F. male female, COPD, chronic obstructive pulmonary disease, CKD, chronic kidney disease.

Table 2
Clinical parameters at the time of analysis.

Clinical parameters median (min-max)	HC n = 50	PAD n = 43	p value
Disease duration, (month)	-	30 (0-212)	
ABI	1.20 (0.97–1.4)	0.61 (0.04–1.12)	0.0001
TBI	0.71 (0.56–1.07)	0.36 (0.09–0.61)	0.0001
Blood pressure - systolic (mmHg)	130 (110–170)	130 (90–210)	0.272
Blood pressure - diastolic (mmHg)	75 (60–95)	70 (55–100)	0.258
Carotid IMT, Max (mm)	0.87 (0.59–1.27)	1.05 (0.65–1.77)	0.0001
Carotid IMT, average (mm)	0.74 (0.48–1.03)	0.87 (0.52–1.27)	0.0001
Carotid Vessel diameter (mm)	5.8 (4.8–8.2)	6.4 (5.0-9.2)	0.0018
VASCUQOL-6	24 (9–24)	14.5 (9–24)	0.0001
Triglycerides (mmol/L)	1.0 (0.6–3.3)	1.2 (0.6–3.4)	0.0248
Cholesterol (mmol/L)	5.7 (3.5–9.2)	4.0 (2.5–7.2)	0.0001
HDL-Cholesterol (mmol/L)	1.8 (1.0-3.1)	1.2 (0.8–1.9)	0.0001
LDL-Cholesterol (mmol/L)	3.5 (1.6–7.2)	2.3 (1.2–5.4)	0.0001
Creatinine (μmol/L)	70 (47–103)	77 (45–156)	0.0933
eGFR Men (ml/min/1.73 m ²)	79.6 (63.4-116.6)	70.2 (30.5-135.1)	0.0473
eGFR Women (ml/min/1.73 m ²)	58.8 (44.2-126.2)	60.6 (38.7-110.8)	0.926
CRP (ng/mL)	1116 (510–3736)	1757 (326–9829)	0.0078
Platelets (×10 ⁹ /L)	210 (108–326)	214 (119–441)	0.175
Neutrophils (×10 ⁹ /L)	2.8 (1.4–4.8)	4.0 (2.5–8.2)	0.0001
Lymphocytes (×10 ⁹ /L)	1.7 (1.0–3.0)	1.9 (0.8–3.5)	0.134
NLR	1.5 (0.6–4.1)	2.2 (1.2–5.3)	0.0003

HC; missing 1; ABI, Creatinine, missing 9; blood pressure systolic/diastolic, missing 2; Triglycerides, eGFR women, missing 15; CRP, missing 8; Platelet, Neutrophil, Lymphocytes, NLR.

PAD; missing 1; carotid IMT, carotid vessel diameter, missing 5; VASCUQOL-6, missing 4; Triglycerides, LDL-cholesterol, Creatinine, missing 2; Cholesterol, HDL-cholesterol, eGFR, missing 3; CRP, blood pressure systolic/diastolic, missing 6; Platelet, Neutrophil, Lymphocytes, NLR.

ABI, ankle-brachial index, TBI, toe-brachial index, IMT, intima media thickness, VASCUQOL-6, vascular quality of life questionnaire-6, HDL, high-density lipoproteins, LDL, low-density lipoproteins, eGFR, estimated glomerular filtration rate, CRP, c-reactive protein, NLR, neutrophil to lymphocyte ratio.

PAD is associated with expansion and altered activation profiles of circulating myeloid cells

We performed a detailed mapping of the immunophenotype of circulating neutrophils, cDCs and monocyte subsets in whole blood from PAD patients and HCs. PAD patients showed a higher proportion of neutrophils within CD45⁺ leukocytes and higher absolute neutrophil counts than HCs (Fig. 1A). Within the antigen-presenting cell (APC; HLA-DR⁺ lineage⁻) compartment, PAD had a lower cDC proportion but higher absolute cDC counts than HCs, suggesting expansion of the APC compartment with a relative reduction in cDC frequency (Fig. 1B).

We previously showed in this cohort that monocyte-subset distributions differ between patients with PAD and HCs¹⁹. Specifically, PAD was associated with a higher proportion of classical monocytes and a lower proportion of non-classical monocytes, whereas intermediate monocyte proportions did not differ between groups. Extending these findings, we show here that absolute counts of all three monocyte subsets were higher in PAD patients than in HCs (Fig. 1C).

To assess activation and migratory status, we evaluated CD162 (PSGL-1) expression on circulating neutrophils and monocyte subsets. The proportion of CD162⁺ cells did not differ between PAD and HCs for neutrophils, classical monocytes, or intermediate monocytes, whereas the proportion of CD162⁺ non-classical monocytes was lower in PAD (Fig. 1D). In contrast, absolute counts of CD162⁺ neutrophils and CD162⁺ classical monocytes were higher in PAD patients, with a similar trend observed for CD162⁺ intermediate monocytes, likely reflecting the overall expansion of these cell populations. CD162⁺ non-classical monocyte counts did not differ between groups (Fig. 1E).

To determine whether the observed differences in immune markers were independent of potential confounders, univariate general linear models were performed with adjustment for age, BMI, and smoking status. As presented in Supplementary Table 2, most of the immune markers assessed remained statistically significantly different between PAD versus HC after adjustment for age, BMI and smoking status.

To further explore the relationship between immune alterations and clinical phenotypes, we assessed associations between neutrophil and monocyte related measures and key indicators of vascular status and systemic inflammation. Classical haematological and immune markers showed only weak, non-significant relationships with vascular structure and haemodynamic severity. Neutrophil counts tended

to correlate inversely with maximal carotid IMT, while neutrophil proportions and classical monocyte count showed borderline negative associations with carotid vessel diameter (Fig. 1F). In contrast, intermediate monocyte counts showed a significant positive correlation with ABI (Fig. 1F).

Together, these results suggest that PAD is associated with a distinct circulating myeloid immune phenotype, marked by neutrophil expansion, increased monocyte subset counts, and altered CD162⁺ cell profiles, largely independent of age, BMI, and smoking status.

Inflammatory pathway activation in PAD neutrophils with unchanged classical monocyte transcriptomes

To determine whether the immunophenotypic alterations observed in PAD were accompanied by disease-associated transcriptional changes, we profiled mRNA expression by RNA sequencing in FACS-sorted neutrophils and classical monocytes from HCs and patients with PAD (HCs, n = 20; PAD, n = 20 for each cell type). Three neutrophil samples from patients with PAD were excluded because of low RNA quality (RIN = 2.6, n = 1) or failure to pass quality control during library preparation (n = 2). Principal component analysis (PCA) and unsupervised clustering separated PAD neutrophils from HC neutrophils, indicating a distinct transcriptional signature in PAD (Fig. 2A–B). Differential expression analysis of 12,275 genes passing expression filtering identified 171 significantly differentially expressed genes (DEGs; false discovery rate [FDR]-adjusted $p < 0.05$) in PAD compared with HCs, including 92 upregulated and 79 downregulated genes (Fig. 2C; Supplementary Excel worksheet DEG).

Next, we performed GSEA using ranked gene lists to identify coordinated pathway-level changes between PAD and HCs. GSEA using GO Biological Process gene sets showed that positively enriched gene sets were mainly related to immune and inflammatory programs, including leukocyte migration and proliferation, IL-6 production, responses to lipids, oxidative stress, TNF, and external stimuli (Fig. 2D). In contrast, negatively enriched gene sets were primarily associated with the aerobic electron transport chain and ATP synthesis (Fig. 2D). The complete list of enriched gene sets is provided in the Supplementary Excel worksheet GO_BP_GSEA.

GSEA using KEGG pathway gene sets also revealed significant pathway-level differences associated with PAD. Positively enriched pathways included T helper 17 (Th17) cell differentiation, Toll-like receptor (TLR), NOD-like receptor (NLR), nuclear factor kappa B (NF- κ B), and Janus kinase–signal transducer and activator of transcription (JAK–STAT) signalling pathways, as well as lipid and atherosclerosis pathways. Conversely, negatively enriched gene sets were associated with autophagy and oxidative phosphorylation (Fig. 2E; Supplementary Excel worksheet KEGG_GSEA). Together, these data suggest that circulating neutrophils from patients with PAD exhibit an inflammatory transcriptional phenotype accompanied by reduced enrichment of mitochondrial oxidative phosphorylation programs.

In contrast, among 13,927 expressed genes passing expression filtering in classical monocytes, we detected no significant DEGs between PAD patients and HCs at FDR < 0.05, and unsupervised analyses

did not reveal group separation (Supplementary Fig. 3).

We next extended our analysis to the epigenetic level by profiling DNA methylation in circulating neutrophils from HCs and patients with PAD. This analysis was exploratory and based on a relatively small sample size; therefore, the findings should be interpreted as hypothesis-generating. DNA methylation was analysed in 12 patients with PAD and 13 HCs. Following quality control and filtering, 521,852 probes were retained for differential methylation analysis. No differentially methylated CpG sites reached statistical significance after correction for multiple testing (adjusted $p < 0.05$). However, 5,178 CpG sites demonstrated nominal statistical significance ($p < 0.05$), of which 1,135 exhibited a delta beta ($\Delta\beta$) difference ≥ 0.15 . All CpG sites, except for 21 CpG sites, were found hypomethylated in PAD compared to HCs (Supplementary Fig. 4). Methylation levels were evaluated for the differentially expressed genes in PAD neutrophils; however, gene expression did not correlate with methylation.

PAD is associated with divergent myeloid cytokine responses: neutrophil hyper-responsiveness and monocyte hypo-responsiveness

Given the inflammatory transcriptional phenotype observed in PAD neutrophils, together with reduced enrichment of mitochondrial oxidative phosphorylation programs, we next examined whether circulating myeloid cells from patients with PAD exhibited altered functional responses. We first assessed intracellular cytokine and chemokine production capacity in whole blood at baseline and after stimulation with LPS or PMA. Following LPS stimulation, neutrophils from patients with PAD produced higher levels of IL-6 and IL-8 than those from HCs, whereas no differences were observed at baseline or after PMA stimulation (Fig. 3A; Supplementary Fig. 5A, Supplementary Fig. 5B). Consistent with this enhanced inflammatory response, plasma IL-6 concentrations were higher in patients with PAD, while plasma IL-8 and IL-1 β concentrations were comparable between patients with PAD and HCs (Fig. 3E). Further, intracellular neutrophil IL-6, but not IL-8, remained significantly different between PAD and HCs after adjustment for age and BMI. Both were not significantly different between the groups after adjustment with smoking status (Supplementary Table 2).

In monocytes, baseline cytokine production was largely similar between groups, except for lower TNF- α production by intermediate monocytes from patients with PAD (Supplementary Fig. 5A). After LPS stimulation, classical monocytes from patients with PAD produced lower levels of IL-6, IL-1 β , and IL-8 than those from HCs (Fig. 3B), while intermediate monocytes produced less IL-6 (Fig. 3C), and non-classical monocytes showed no significant differences (Fig. 3D). Following PMA stimulation, classical monocytes from patients with PAD showed reduced IL-1 β production, whereas non-classical monocytes showed reduced IL-8 production (Supplementary Fig. 5B). Following adjustment for age, BMI and smoking status, most of the altered cytokine production responses from these monocyte population remained statistically significant except for IL-6 and IL-1 β production by classical monocyte which failed to be significant after adjustment for age (Supplementary Table 2).

To further assess cytokine production capacity, we used LPS stimulated *in vitro* 3D PBMC cultures. Baseline unstimulated cultures show comparable IL-1 β and TNF- α levels in between the groups. However, following LPS stimulation, PBMCs from PAD patients produced higher levels of IL-1 β but lower levels of TNF- α in culture supernatants than PBMCs from HCs (Fig. 3F-G). Nevertheless, these differences in TNF- α and IL-1 β production were no longer significant after adjustment for age, BMI, or smoking status (Supplementary Table 2).

Collectively, these findings indicate that circulating myeloid cells from PAD patients show stimulus-specific functional alterations, characterised by enhanced neutrophil IL-6 and IL-8 responses to LPS, attenuated cytokine production by monocyte subsets, and altered PBMC cytokine responses that appear partly influenced by clinical confounders.

Impaired phagocytosis, reduced neutrophil ROS, and elevated MPO characterize myeloid dysfunction in PAD

Having observed divergent cytokine responses in PAD neutrophils and monocyte subsets, we next assessed whether other core myeloid effector functions were altered in PAD, including phagocytosis, reactive oxygen species (ROS) production, myeloperoxidase (MPO) content, and neutrophil extracellular trap (NET) associated markers. Because phagocytic clearance contributes to the resolution of inflammation, we quantified bacterial phagocytosis using fluorescent *E. coli* bioparticles in whole blood from patients with PAD and HCs. Neutrophils and classical monocytes from PAD patients exhibited reduced phagocytic capacity compared to the HCs (Fig. 4A), and non-classical monocytes showed similar but more moderate impairment (Fig. 4A). We next quantified intracellular ROS in circulating neutrophils and monocyte subsets by flow cytometry. Neutrophils from patients with PAD had lower intracellular ROS levels than those from HCs (Fig. 4B), whereas intracellular ROS levels were similar across monocyte subsets and in total monocytes (Supplementary Fig. 6). Most of these differences remained statistically significant after adjustment for age, BMI and smoking status except for age-adjusted classical monocyte phagocytosis and adjusted non-classical monocyte phagocytosis (Supplementary Table 2).

We next quantified MPO, an azurophilic granule enzyme involved in inflammation, phagocytosis, and NET formation. The proportion of MPO⁺ cells did not differ between patients with PAD and HCs across neutrophils or monocyte subsets (Fig. 4C). In contrast, absolute counts of MPO⁺ cells were higher in patients with PAD among neutrophils and classical and intermediate monocytes, reflecting expansion of these populations, whereas MPO⁺ non-classical monocyte counts were unchanged (Fig. 4D). Plasma soluble MPO concentrations were also higher in PAD than in HCs (Fig. 4E), and plasma MPO tended to correlate positively with ABI (Fig. 4F). The differences in these parameters between PAD and HCs remained statistically significant after adjustment for age, BMI and smoking status except for MPO⁺ intermediate monocyte counts which failed statistical significance after adjustment for smoking status (Supplementary Table 2).

Finally, to assess circulating NET-associated markers, we quantified citrullinated histone H3 (CitH3) and MPO–DNA complexes in plasma by ELISA. Neither CitH3 nor MPO–DNA levels differed between patients with PAD and HCs (Fig. 4G).

Together, these data suggest that PAD is characterised by quantitative expansion of circulating myeloid populations together with qualitative functional alterations, including impaired phagocytosis, reduced neutrophil intracellular ROS, and elevated circulating soluble MPO, without evidence of increased circulating NET-associated markers.

Discussion

In the present study, we provide an integrated characterisation of circulating myeloid cells in PAD by combining immunophenotyping, functional assays, transcriptomic profiling of neutrophils and monocytes, and exploratory epigenetic analysis of circulating neutrophils. We show that PAD is marked by expansion of circulating neutrophils and monocyte subsets, accompanied by distinct cell type-specific functional alterations. Several of these differences persisted after adjustment for age, BMI, and smoking status, suggesting that they are not solely explained by major demographic or clinical confounders.

PAD was associated with distinct cell type-specific myeloid alterations. Neutrophils displayed an inflammatory, TLR4-responsive phenotype, with increased LPS-induced IL-6 and IL-8 production, reduced phagocytosis, and lower intracellular ROS. In contrast, monocytes showed reduced cytokine production and impaired phagocytosis despite increased absolute counts. Transcriptomic analysis revealed enrichment of inflammatory signalling pathways and reduced enrichment of mitochondrial, oxidative phosphorylation, and autophagy programs in PAD neutrophils, whereas classical monocytes showed no significant differential gene expression. Together, these findings indicate systemic myeloid remodelling in PAD, characterised by neutrophil transcriptional activation and broader impairment of innate immune effector functions.

These findings extend human data identifying neutrophils as active contributors to vascular inflammation and atherothrombosis^{20,21}. The enhanced PAD neutrophil IL-6/IL-8 response to LPS aligns with the observed elevation of plasma IL-6 and with transcriptomic enrichment of inflammatory and lipid-response pathways. Clinically, these signatures resonate with reports that higher neutrophil counts and NLR associate with PAD severity and adverse outcomes^{12,14,22}. Our data on higher absolute count for MPO⁺ neutrophils together with elevated soluble MPO supports systemic neutrophil activation and degranulation in PAD, which is consistent with links between circulating MPO and plaque vulnerability and adverse events in patients with PAD and CAD^{22,23}. By contrast, circulating NET-associated markers (CitH3 and MPO–DNA) were not increased in PAD patients. These results are in line with the findings of Buso et al., who also reported no difference in MPO–DNA levels between PAD patients and HCs²², and may reflect lesion-localized or transient NETosis, pre-analytical factors, or medication effects. In particular, statins can reduce circulating NET-related markers in patients with coronary artery disease²⁴,

and most PAD patients in the present cohort were receiving statin therapy. Thus, our data suggest altered neutrophil granule associated inflammatory activity rather than overt systemic NET release.

Several mechanisms may link our cellular and transcriptomic findings. Ischemia–reperfusion, a common feature of PAD, can prime neutrophils for exaggerated effector responses and tissue injury²⁵. In addition, PSGL-1 interactions with P-selectin on activated endothelium and platelets may promote neutrophil and monocyte adhesion, priming, and recruitment^{26,27}. Our CD162 data suggest that increased numbers of circulating CD162⁺ neutrophils and CD162⁺ classical monocytes in PAD mainly reflect expansion of these populations rather than increased per-cell CD162 expression. Thus, while these findings may indicate greater availability of PSGL-1-expressing myeloid cells for vascular recruitment, direct PSGL-1 activation cannot be inferred without CD162 MFI or functional adhesion assays.

At the transcriptomic level, PAD neutrophils showed enrichment of inflammatory pathways, including TLR/NLR/NF- κ B and JAK–STAT signalling, together with reduced enrichment of mitochondrial electron transport, oxidative phosphorylation, and autophagy programs. These changes are consistent with altered neutrophil immunometabolism and defective cargo handling, providing a plausible link to reduced phagocytosis and lower intracellular ROS^{28–32}. Differences from previous report of increased whole-blood ROS in PAD patients with CLTI may reflect disease severity, assay design, sample type, or statin therapy, which can reduce neutrophil ROS³³. Enrichment of lipid/atherosclerosis and Th17-related pathways further suggests that PAD neutrophils are shaped by a lipid-rich inflammatory vascular milieu that may contribute to endothelial injury and impaired resolution of inflammation³⁴.

Notably, the differentially expressed genes identified in PAD neutrophils did not show methylation differences in our exploratory DNA methylation analysis. This suggests that the transcriptional changes observed in PAD neutrophils are unlikely to be explained by DNA methylation changes in this dataset. Instead, these transcriptional changes in neutrophils between PAD and HC may be shaped by other regulatory mechanism affecting transcription factor activity such as histone modifications, non-coding RNAs, post-transcriptional regulation, or stimulus-dependent signalling pathways. Given the modest sample size and exploratory nature of the methylation analysis, these findings should be interpreted cautiously and validated in larger studies using integrated multi-omics approaches.

Monocytes presented a distinct pattern in our study. Despite increased absolute counts and subset shifts, as observed here and reported by others in PAD patients^{19,35,36}, monocytes from PAD patients exhibited reduced cytokine production and impaired phagocytosis, consistent with attenuated inflammatory responsiveness. Classical and intermediate monocytes produced lower levels of IL-6, IL-1 β , and IL-8 after TLR4 or PKC stimulation, and classical monocytes showed the most prominent reduction in phagocytic capacity. These findings indicate stimulus-specific monocyte hyporesponsiveness and attenuated effector function in PAD. We previously observed higher frequencies of PD-L2⁺ cells within all monocyte subsets in patients with PAD¹⁹. PD-L2 upregulation is compatible with differentiation toward immunoregulatory or tissue-resident macrophage-like states under Th2-skewed cues, which may help explain the attenuated cytokine responses observed *ex vivo*³⁷. In contrast

to neutrophils, classical monocytes showed no significant DEGs at resting state, despite functional alterations at the cellular level. This supports a conditional, context-dependent program that may be shaped *in vivo* by chronic ischemia, oxidative stress, platelet–leukocyte interactions, and anti-inflammatory or tissue-remodelling cues. Consistent with this interpretation, recent single-cell profiling of human carotid and femoral plaques reported site-specific myeloid composition, with femoral lesions enriched for resident macrophage-like, non-inflammatory foam cell-like, and LYVE1-high macrophage populations³⁸.

Exploratory correlation analyses showed weak and largely non-significant associations between circulating myeloid markers and vascular structural or haemodynamic measures and should therefore be interpreted cautiously. Overall, the observed trends did not support a simple relationship between myeloid activation and PAD severity but instead suggest that circulating myeloid phenotypes may reflect systemic inflammatory or remodelling processes only partly captured by conventional vascular assessments. Larger longitudinal studies are needed to determine whether these immune alterations relate to disease stage, plaque activity, limb ischemia, treatment effects, or future vascular outcomes.

Given that PAD is highly age-associated and that patients in this cohort were older than HCs, adjustment analyses were important for distinguishing disease-associated immune alterations from major host-related confounders. Collectively, our findings suggest that PAD is associated with systemic myeloid immune remodelling, characterised by expansion of circulating myeloid populations, neutrophil transcriptional activation, altered neutrophil granule associated inflammatory activity, and impaired innate immune effector responses in the context of aging and inflammatory burden.

An important implication of our findings is that systemic myeloid immune remodelling was evident in PAD despite the widespread use of cardiovascular medications and risk-factor management, including lipid-lowering therapy. Although such treatments may improve symptoms and reduce conventional cardiovascular risk factors, patients with PAD may continue to exhibit a residual inflammatory burden that is not fully captured by clinical measures alone. In this context, alterations in myeloid cell counts, proportions, and effector functions may represent a cellular mechanism underlying this persistent inflammatory state. However, these immune alterations should be interpreted within the treatment context of the cohort, as cardiovascular medications, particularly statins, may modulate neutrophil ROS production, MPO release, NET-associated markers, and inflammatory responses. Thus, the observed expansion of circulating myeloid populations, neutrophil inflammatory priming, altered MPO-associated activity, impaired phagocytosis, and monocyte hyporesponsiveness may reflect persistent myeloid remodelling in treated PAD rather than untreated disease biology alone.

This study has several strengths. We provide an integrated characterization of circulating myeloid cells in PAD by combining immunophenotyping, *ex vivo* functional assays, plasma protein measurements, transcriptomic profiling, and exploratory epigenetic analysis. This multi-layered approach enabled assessment of both quantitative changes in immune-cell populations and qualitative alterations in myeloid-cell function. We also evaluated complementary neutrophil and monocyte effector functions,

including cytokine production, phagocytosis, intracellular ROS, MPO, and circulating NET-associated markers, supported by well-established flow cytometry panels. In addition, adjustment analyses for age, BMI, and smoking status indicated that several observed immune differences were not solely explained by these major clinical confounders.

Several limitations should also be acknowledged. The cross-sectional design limits causal inference and prevents assessment of whether the observed myeloid alterations contribute to PAD progression or reflect established vascular disease. The modest sample size, particularly for some functional assays, may have reduced statistical power and limited the number of covariates that could be included reliably in adjusted models. Variable data availability across assays also limited robust multivariate analyses integrating immune, transcriptomic, and clinical variables. PAD patients were older than HCs, and although adjustment analyses were performed, residual confounding cannot be excluded. Most PAD patients were receiving cardiovascular medications, including statins, which may influence neutrophil ROS production, MPO release, NET-associated markers, and inflammatory responses. In addition, CD162 MFI was not assessed; therefore, CD162-related findings should be interpreted as changes in CD162⁺ cell frequency and absolute counts rather than direct evidence of altered PSGL-1 expression intensity or activation. Finally, the transcriptomic, epigenetic, and vascular correlation analyses were exploratory and may not fully capture immune-cell states within vascular lesions or ischemic tissues. Larger longitudinal studies are needed to determine whether these circulating myeloid signatures relate to disease stage, plaque activity, limb ischemia, treatment response, or future cardiovascular outcomes.

Conclusion

In conclusion, this study identifies systemic myeloid immune remodelling as a feature of PAD in the context of aging. PAD was characterised by expansion of circulating neutrophils, cDCs, and monocyte subsets, together with distinct functional alterations in neutrophils and monocytes. Neutrophils displayed enhanced inflammatory responsiveness to LPS, elevated MPO-associated inflammatory activity, reduced phagocytosis, and lower intracellular ROS, accompanied by transcriptomic enrichment of inflammatory pathways and reduced enrichment of mitochondrial and autophagy-related programs. In contrast, monocytes showed attenuated cytokine production and impaired phagocytic capacity despite increased absolute counts. These findings suggest that PAD is not simply associated with generalized myeloid activation, but rather with cell type-specific remodelling of innate immune function. Future longitudinal studies should determine whether these circulating myeloid signatures contribute to PAD progression, reflect vascular aging and ischemic burden, or predict clinical outcomes and treatment responses. Such studies should ideally include adjusted analyses across the spectrum of PAD severity, from intermittent claudication to chronic limb-threatening ischemia, and integrate tissue-level profiling of atherosclerotic lesions and lesion-adjacent blood to define neutrophil mechanisms within the vascular microenvironment.

Declarations

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

SK and AKK: Conceptualization of the study, Writing – original draft. SK, FP, RR, SAF and ID: Performed experiments SK, FP, RR, SAF, JC, MMZ, ID and AKK: Data collection and data analysis MHP, HH and MD: Study participant recruitment and data collection SK, SAF, MHP, PM, MMZ, ID, IR, AS and AKK: Data interpretation SK, FP, RR, SAF, JC, MHP, HH, MD, MMZ, PM, ID, IR, AS, AKK: reviewing and editing the manuscript MMZ, AS and AKK: supervision AKK, HH and SK: Funding acquisition All authors were involved in reviewing and editing the manuscript and approved the final draft of manuscript, including the authorship list.

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Data Availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

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Figures

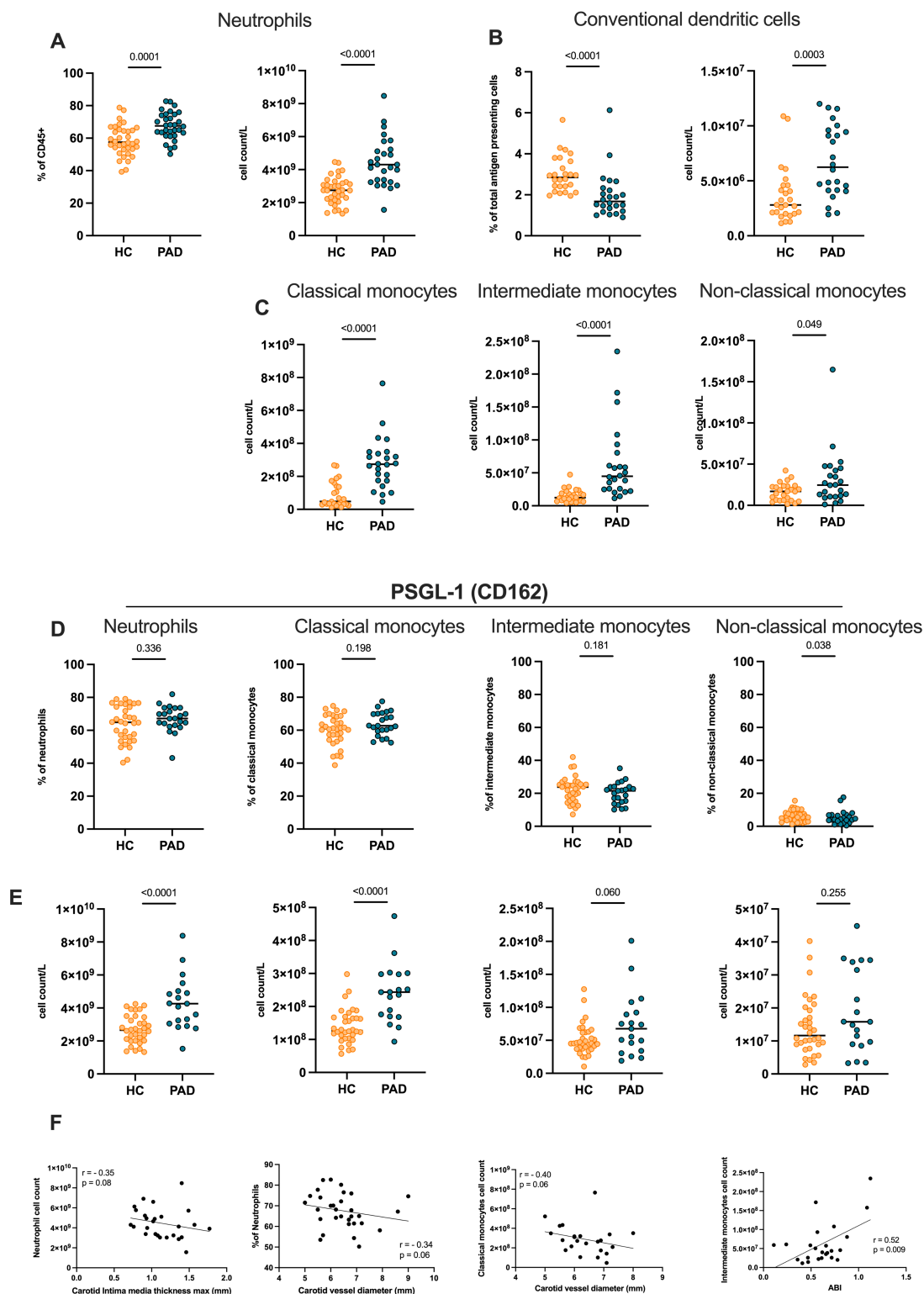


Figure 1

PAD is associated with expansion and altered activation profiles of circulating myeloid cells.

Immunophenotyping of neutrophils, cDCs and monocyte subsets in whole blood using flow cytometry.

(A) Proportion of neutrophils in PAD patients (n=32) and HCs (n=36), and absolute neutrophil counts (cell count/L) in PAD patients (n=27) and HCs (n=36). **(B)** Proportion and absolute cDC counts in PAD patients (n=24) and HCs (n=26). **(C)** Absolute counts of monocyte subsets in PAD patients (n=24) and

HCs (n=26). **(D)** Proportion of CD162⁺ (PSGL-1⁺) neutrophils and monocyte subsets in PAD patients (n=23) and HCs (n=34), and **(E)** absolute counts of CD162⁺ (PSGL-1⁺) neutrophils and monocyte subsets in PAD patients (n=19) and HCs (n=34). **(F)** Associations of neutrophil count (n=27), proportion of neutrophils (n=32), classical monocyte count (n=24) and intermediate monocyte count (n=24) with carotid IMT and carotid vessel diameter, and ABI in PAD patients. Spearman's rank correlation coefficient was used to assess associations between neutrophil and monocyte subsets from PAD patients with clinical parameters. Mann-Whitney U tests were used to compare PAD patients and HCs. Data are presented in scatter dot plots; horizontal bars indicate the median. P-values are shown in the graphs. PAD, peripheral artery disease; HC, healthy controls; cDCs, conventional dendritic cells; PSGL-1, P-selectin glycoprotein ligand-1; IMT, intima-media thickness; ABI, ankle-brachial index.

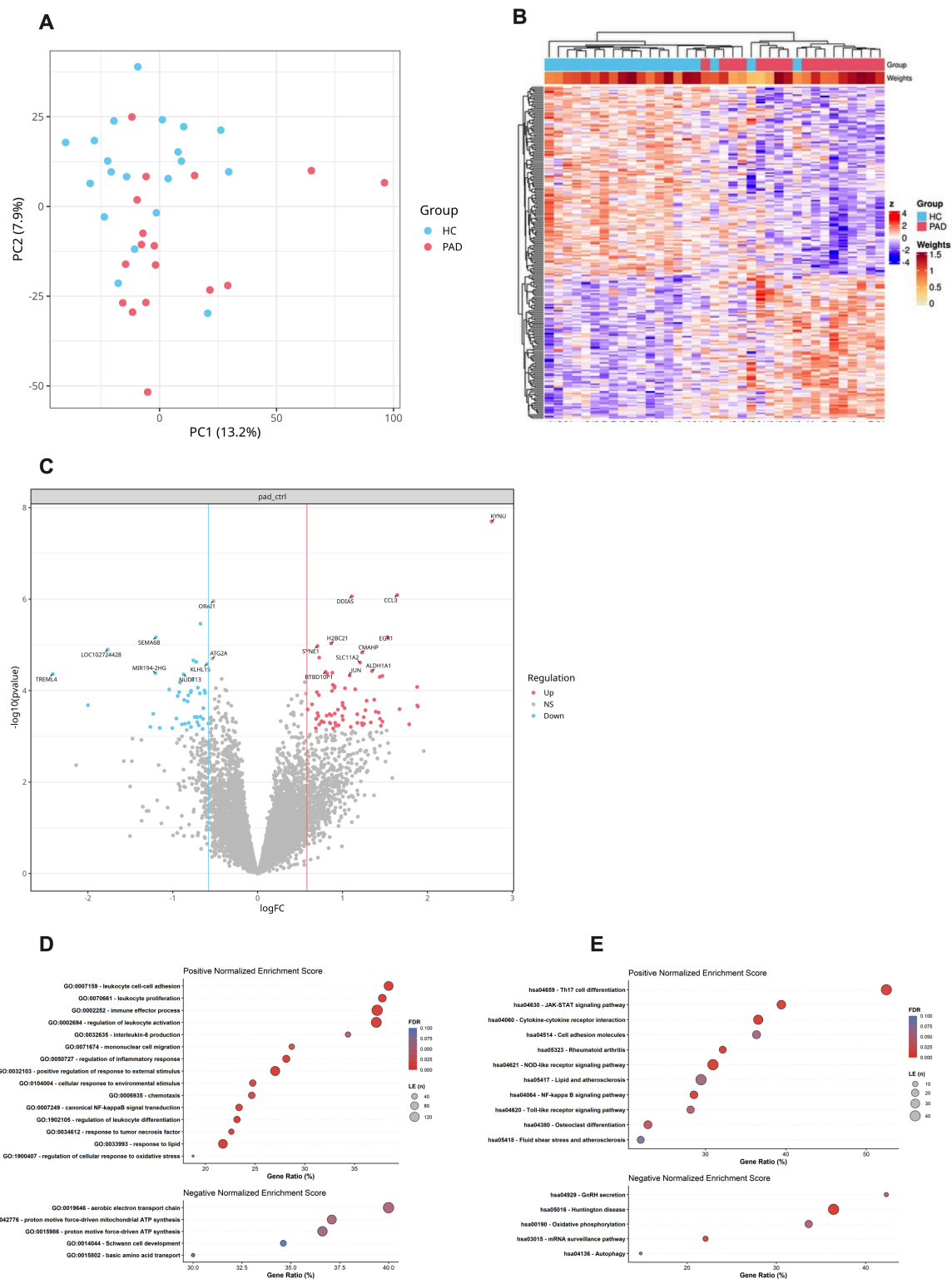


Figure 2

RNA-sequencing reveals distinct neutrophil transcriptomic profiles in PAD. RNA-sequencing was performed on FACS-sorted circulating neutrophils from patients with PAD (n = 17) and HCs (n = 20). **(A)** Principal component analysis (PCA) showing global transcriptomic variation between PAD and HC neutrophils. **(B)** Heatmap with unsupervised hierarchical clustering illustrating separation of PAD and HC neutrophil transcriptomes. **(C)** Volcano plot showing differentially expressed genes between PAD and

HCS. **(D, E)** Gene set enrichment analysis (GSEA) showing pathways positively or negatively enriched in PAD neutrophils relative to HCs, including **(D)** Gene Ontology (GO) biological processes and **(E)** Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. PAD, peripheral artery disease; HC, healthy controls; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

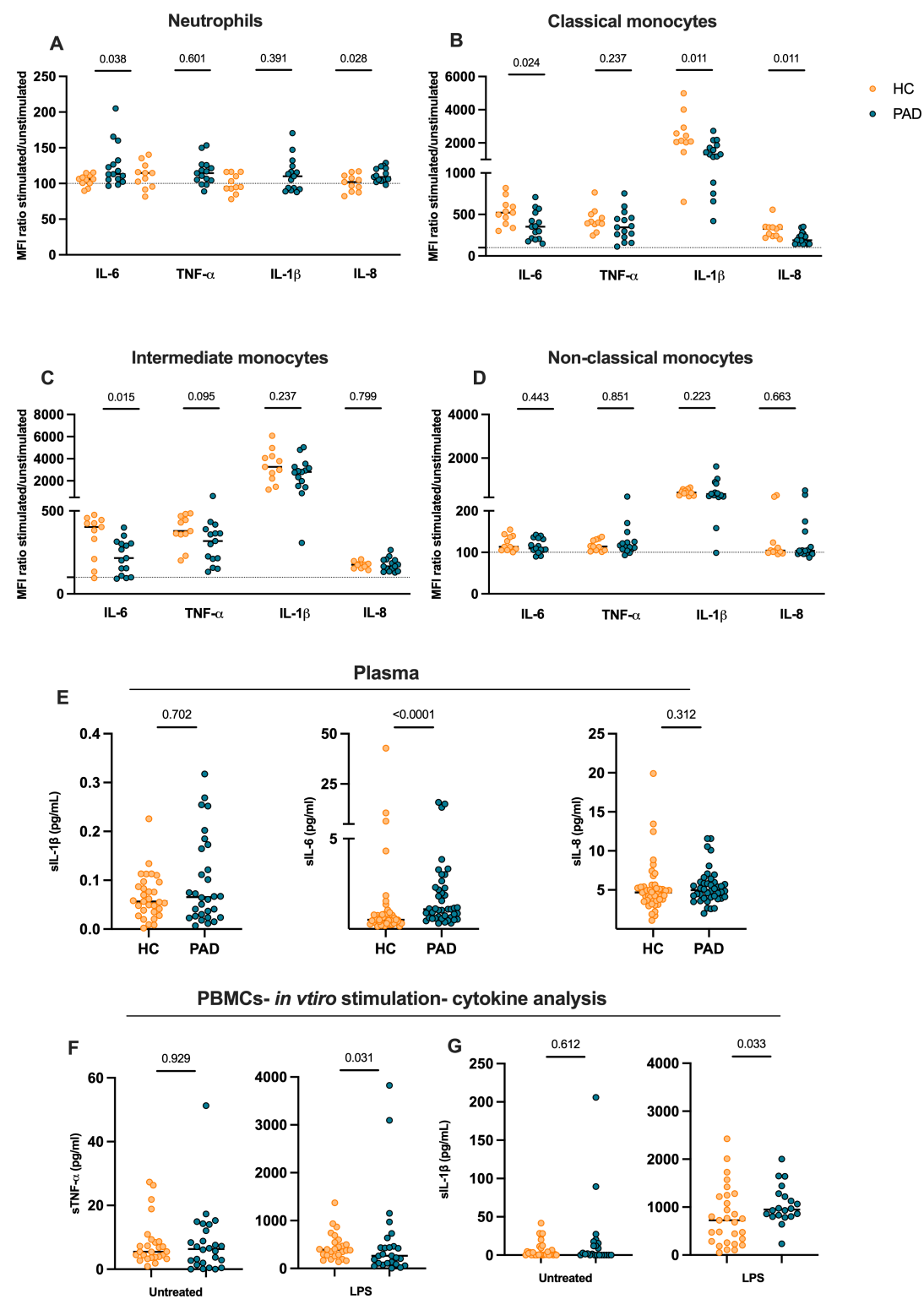


Figure 3

PAD is associated with divergent myeloid cytokine responses: neutrophil hyper-responsiveness and monocyte hypo-responsiveness. Intracellular cytokine/chemokine staining following LPS stimulation in whole blood analysed by flow cytometry in PAD patients (n=15) and HCs (n=11). **(A)** Neutrophils, **(B)** classical monocytes, **(C)** intermediate monocytes and **(D)** non-classical monocytes. MFI of stimulated cells for each sample was normalized against the unstimulated control. **(E)** Plasma levels of pro-inflammatory mediators (IL-1 β , IL-6, IL-8) quantified by MSD assay in PAD patients (n=42) and HCs (n=44). **(F)** TNF- α levels measured by ELISA in PBMC supernatants after 48 hours of stimulation with or without LPS in PAD patients: untreated n=26, LPS n=26 and HCs: untreated n=26, LPS n=26. **(G)** IL-1 β levels measured by ELISA in PBMC supernatants after 48 hours of stimulation with or without LPS in PAD patients: untreated n=23, LPS n=20 and HCs: untreated n=30, LPS n=29. Mann-Whitney U tests were used to compare PAD patients and HCs. Dash line on plots A-D represents baseline levels of analysed cytokines. Data are presented in scatter dot plots; horizontal bars indicate the median. P-values are shown in the graphs. PAD, peripheral artery disease; HC, healthy controls; LPS, lipopolysaccharide; MFI, median fluorescence intensity; PBMCs, peripheral blood mononuclear cells; TNF- α , tumour necrosis factor alpha; IL-1 β , interleukin-1 beta.

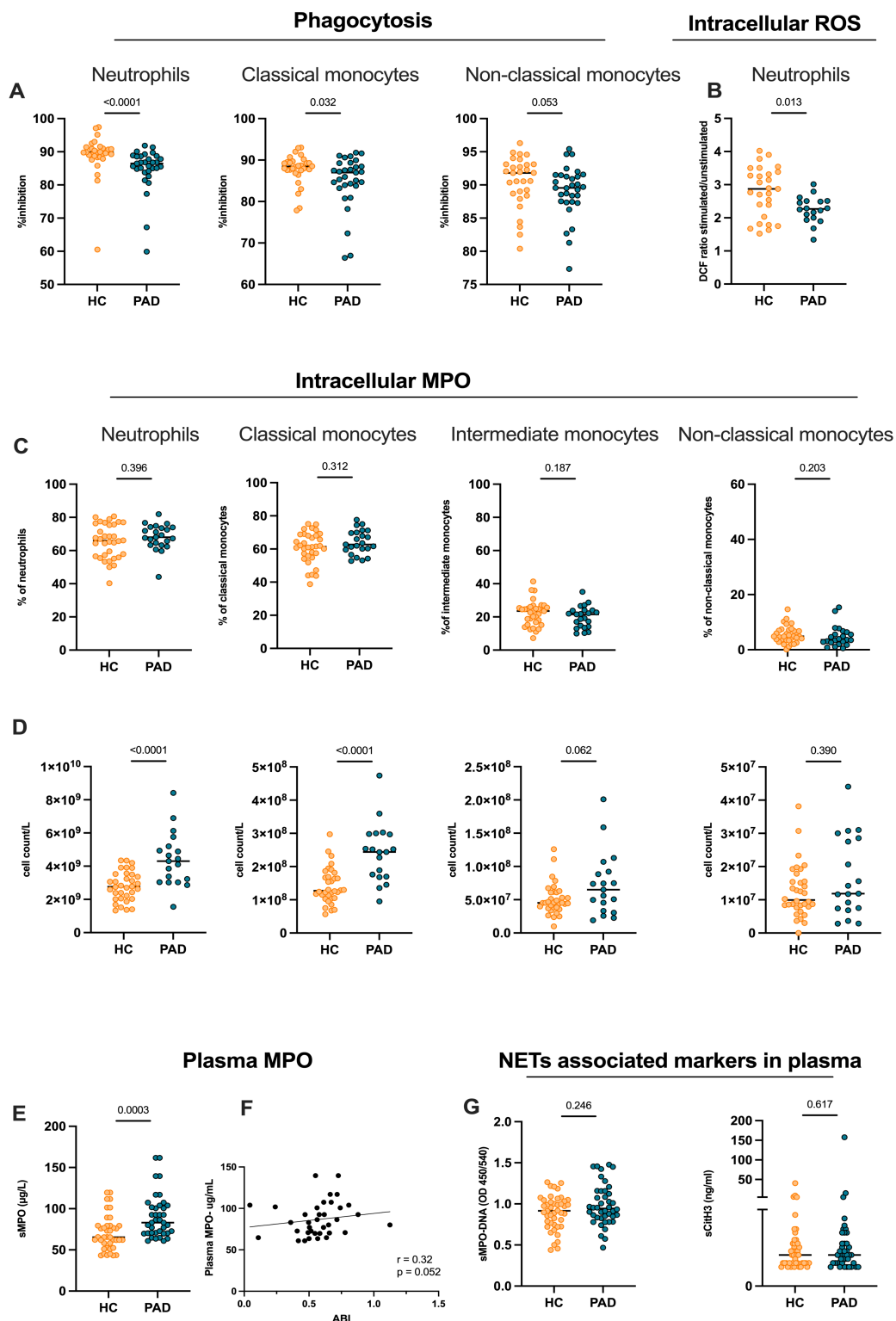


Figure 4

Impaired phagocytosis, reduced neutrophil ROS, and elevated MPO characterize myeloid dysfunction in PAD (A) Phagocytosis assay in whole blood analysed by flow cytometry in PAD patients (n=31) and HCs (n=30). Percentage phagocytosis inhibition was determined as $(MFI_{positive} - MFI_{inhibitory}) / MFI_{positive}$. (B) Intracellular ROS in neutrophils measured by flow cytometry in PAD patients (n=18) and HCs (n=27). MFI of stimulated cells for each sample was normalized against the unstimulated control. Flow cytometry

analysis of intracellular MPO: **(C)** proportions of MPO⁺ neutrophils and monocyte subsets in PAD patients (n=23) and HCs (n=34), and **(D)** absolute counts of MPO⁺ neutrophils and monocyte subsets in PAD patients (n=19) and HCs (n=34). **(E)** Plasma MPO levels quantified by ELISA in PAD patients (n=39) and HCs (n=40). **(F)** Associations of plasma MPO (n=39) with ABI in PAD patients. **(G)** Plasma NET-associated markers (MPO-DNA and CitH3) quantified by ELISA in PAD patients (n=42) and HCs (n=44). Spearman's rank correlation coefficient was used to perform association between plasma MPO and ABI. Mann-Whitney U tests were used to compare PAD patients and HCs. Data are presented in scatter dot plots; horizontal bars indicate the median. P-values are shown in the graphs. PAD, peripheral artery disease; HC, healthy controls; MFI, median fluorescence intensity; MPO, myeloperoxidase; IC, intracellular; ROS, reactive oxygen species; NETs, neutrophil extracellular traps; CitH3, citrullinated histone H3; DNA, deoxyribonucleic acid; ABI, ankle-brachial index.

Supplementary Files

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