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BRIEF REPORT

Dual-Pathway Inhibition with Rivaroxaban and Low-Dose Aspirin Does Not Alter Immune Cell Responsiveness and Distribution in Patients with Coronary Artery Disease

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ABSTRACT

Introduction: Cardiovascular diseases (CVD) are the leading cause of death globally. Inflammation is an important driver of CVD where tissue damage may lead to the formation of deadly thrombi. Therefore, antithrombotic drugs, such as platelet inhibitors, are crucial for secondary risk prevention in coronary artery disease (CAD) and peripheral artery disease (PAD). For severe forms of the disease, dual-pathway inhibition (DPI) where low-dose aspirin is combined with rivaroxaban has shown improved efficacy in reducing cardiovascular mortality.

Methods: Given this greater improvement in mortality, and the importance of inflammation in driving atherosclerosis, the potential for off-target inflammation-lowering effects of these drugs was evaluated by looking at the change in immune cell distribution and responsiveness to ex vivo lipopolysaccharide (LPS) stimulation after 3 months of DPI in patients with CAD.

Results: We observed no changes in whole blood or peripheral blood mononuclear cell (PBMC) immune cell responsiveness to LPS after 3 months of DPI. Additionally, we did not observe any changes in the distribution of total white blood cells, monocytes, neutrophils, lymphocytes, or platelets during the study course. Signs of systemic inflammation were studied using Olink proteomics in 33 patients with PAD after 3 months of DPI. No changes were observed in any of the inflammatory proteins measured after the treatment period, suggesting that the state of chronic inflammation was not altered in these subjects.

Conclusion: Three months of DPI does not result in any meaningful change in immune cell responsiveness and distribution in patients with CAD or PAD.

Trial Registration: ClinicalTrials.gov ID: NCT05210725

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Keywords: Inflammation; Cardiovascular diseases; Platelet aggregation inhibitors; Factor Xa inhibitors; Immunity

Key Summary Points

Why carry out this study?

Inflammation is an important driver of cardiovascular diseases where tissue damage may lead to the formation of deadly thrombi.

Dual-pathway inhibition (aspirin combined with rivaroxaban) has shown improved efficacy in reducing cardiovascular mortality for severe forms of coronary and peripheral artery disease.

This study investigated whether the beneficial effects of dual-pathway inhibition were a result of reducing systemic inflammation.

What was learned from the study?

Three months of dual-pathway inhibition does not alter immunophenotypes of patients with cardiovascular diseases.

The clinical benefit of dual-pathway inhibition over aspirin alone is more likely related to the intensified level of anticoagulation, and not to the inhibition of inflammatory pathways.

INTRODUCTION

The leading cause of mortality globally is cardiovascular disease (CVD) [1]. Although there are many underlying causes for CVD, coronary artery disease (CAD) and peripheral artery disease (PAD) are primarily caused by atherosclerotic plaques within arterial walls. Tissue injury associated with atherosclerosis, particularly plaque rupture, can activate platelets and proteins of the coagulation cascade, resulting in the formation of thrombi that restrict blood flow through the artery, potentially leading to ischemic tissue damage. Therefore, patients are generally prescribed antithrombotic medication to reduce complications associated with thrombi formation.

Amongst the most widely prescribed antithrombotic drugs is aspirin, which reduces hemostasis by inhibiting thromboxane production in platelets and thereby reduces their activation and aggregation. Although this course of treatment is effective, many patients will still experience recurrent events every year [2]. The COMPASS trial demonstrated that for more complex cases of recurrent cardiovascular events, aspirin combined with low-dose rivaroxaban improved the event-free survival when compared to monotherapy of either drug [3]. Rivaroxaban works by directly inhibiting factor X, an important rate-limiting enzyme in the coagulation cascade. Therefore, combined aspirin and rivaroxaban serves as a method of dual-pathway inhibition (DPI) with regards to thrombus formation.

Much of the benefit of DPI is likely the result of reducing the formation of blood clots within the arteries of patients. However, apart from thrombus formation, a state of low-grade chronic inflammation is known to play an important role in CVD onset and progression [4]. Aspirin has well-studied modulatory effects on inflammation and immune cell function [5, 6]. Furthermore there is cross talk between proteins of the coagulation cascade and inflammatory cells, most notably thrombin and PAR2 receptors on monocytes/macrophages [7, 8]. This opens the possibility for indirect effects of rivaroxaban on immune cell modulation. Therefore, we hypothesize that part of the additive benefit of DPI is via off-target by modulation of immune cell-driven inflammation. This study aimed to investigate whether the beneficial effects of DPI were a result of reducing systemic inflammation, and if these changes were accompanied by alterations in immune cell responsiveness and circulating distribution.

METHODS

Study Design

An explorative interventional trial investigating the effect of DPI on monocyte-driven inflammation was initiated by and conducted in the Radboud University Medical Center (Nijmegen, the Netherlands). Approval of the Medical Research Ethics Committee Oost-Nederland (file number 2021-13291) and the local institutional review board was obtained. This study was conducted in accordance with the latest revision of the Helsinki Declaration of 1964 and Good Clinical Practice regulations and was registered at ClinicalTrials.gov on January 27, 2022 (registration number NCT05210725). Written informed consent was obtained from all participants.

Participants

Patients with stable CAD and an indication for single antiplatelet therapy according to the leading international guidelines [9] were recruited at our outpatient clinic. Patients were considered eligible when they were (1) currently treated with aspirin (80–100 mg once daily) monotherapy, (2) at high risk of developing recurrent vascular events based on a SMART risk score $\geq$ 20%, (3) at least 1 year after myocardial infarction or suffering from multivessel CAD, and (4) aged $\geq$ 16 years. Exclusion criteria were concomitant use of immunosuppressant/anti-inflammatory therapies and known contraindications to rivaroxaban including hypersensitivity, at significant risk for major bleeding, severe hepatic disease, severe kidney failure (estimated glomerular filtration rate $<$ 15 ml/min or requiring dialysis), severe heart failure (ejection fraction $<$ 30% or New York Heart Association class III or IV symptoms), or concomitant treatment with medication with a strong pharmacokinetic interaction with rivaroxaban.

Procedures

Eligible patients visited the hospital three times, once at baseline and twice during follow-up (after 4 and 12 weeks of DPI treatment, respectively).

Baseline

At baseline (T0), written informed consent was obtained. Data regarding demographics, lifestyle, medical history, and medication use (including recent vaccinations, at most 1 month before screening) were recorded using standardized case report forms and electronic patient files. Measurement of blood pressure, height, weight, and hip-waist circumference took place. Venous blood samples were collected. Finally, participants were prescribed rivaroxaban 2.5 mg twice daily for a 12-week period and follow-up visits were scheduled.

Follow-up

Patients were scheduled for follow-up after 4 weeks (T1) and 12 (T2) weeks of DPI treatment. During follow-up visits, adverse events were reported, and medication adherence was evaluated by interview. If medication adherence was below 80%, rivaroxaban was discontinued, and participants were excluded from further study participation. Venous blood samples were collected.

Whole Blood Stimulation

A 100-µl sample of whole blood was added to each round-bottomed well of a 96-well plate along with 400 µl RPMI 1640 Medium ("Dutch modification" containing 11 mM glucose; Thermo-Fischer, Waltham, MA, USA) supplemented with 10 µg/ml gentamicin, 2 mM GlutaMAX and 1 mM pyruvate, or 400 µl culture medium supplemented with 12.5 ng/ml for a final concentration of 10 ng/ml of *Escherichia coli* lipopolysaccharide (LPS) (serotype 055:B5 Sigma-Aldrich, St. Louis, MO). Blood was incubated at 37 °C and 5% CO_2 for 24 h, after which supernatants were collected and stored at − 80 °C until cytokine assessment.

Peripheral Blood Mononuclear Cell Isolation and Stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of study participants by dilution in phosphate-buffered saline (PBS) and density-gradient centrifugation using

Ficoll-Paque (GE healthcare, Chicago, IL, USA). PBMCs were washed three times with cold PBS and resuspended in RPMI 1640 medium. For stimulations experiments, 5×10^5 PBMCs were seeded in each round-bottomed well of a 96-well plate (Corning, NY, USA) and stimulated for 24 h with either culture medium or culture medium supplemented with 10 ng/ml of *E. coli* LPS (serotype 055:B5 Sigma-Aldrich, St. Louis, MO, USA) at 37 °C and 5% CO_2. After incubation for 24 h, supernatants were stored after plate centrifugation at − 80 °C until cytokine assessment.

Cytokine Measurements

Levels of tumor necrosis alpha (TNFα) and interleukin-6 (IL-6) were measured in supernatants using the IL-6 and TNFα DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA).

Immune Cell Measurements via Sysmex

Complete blood counts were performed on EDTA whole blood and PBMC fractions after Ficoll isolation, on the Sysmex XN-450 hematology analyzer (Sysmex America Inc, Lincolnshire, IL, USA).

Olink Analysis in Patients with PAD

An additional cohort of 33 patients with PAD, a subset of the DUAL-PAD study (NCT04218656) [10], were included and assessed for circulating plasma protein expression. Circulating plasma protein expression both at baseline and after 3 months of DPI was assessed using the commercially available multiplex proximity extension assay from Olink® Proteomics AB (Uppsala, Sweden). The Target 96 Inflammation Panel was run in which 96 inflammatory proteins were measured.

Statistical Analysis

Since this is exploratory research, detailed sample size calculation is not appropriate. We aimed to include 15–20 patients based on the average LPS-induced TNFα production in patients with symptomatic atherosclerosis [11], and an expected 20% increase in cytokine production capacity from baseline (aspirin) to 3 months of DPI (aspirin with rivaroxaban). Statistical testing was performed by using the Wilcoxon matched pairs signed rank test. For volcano plots, data were analyzed by Mann–Whitney test; the Benjamini–Hochberg procedure was employed to correct multiple testing errors. False discovery rate (FDR)-adjusted p values smaller than 0.05 were considered statistically significant. Statistical analysis and data visualization were performed with Graphpad Prism v9.3.1 (GraphPad software, La Jolla, CA) or R/Bioconductor (https://www.R-project.org/).

RESULTS

Patient Characteristics

Between March 2022 and April 2022, 16 patients with CAD were enrolled. Medication adherence was above 80% for all participants. Three participants dropped out during the study course because of (1) side effects of rivaroxaban, (2) development of atrial fibrillation with need for more intensive antithrombotic treatment, and (3) active COVID-19 during last episode of study, with high risk of affecting study outcomes. Patient characteristics are shown in Table 1. The median age of the participants was 72 years and 31% were female. The median age of the additional cohort of 33 patients with PAD [10] was 67 and 33% were female.

DPI Does Not Change Ex Vivo Immune Cell Responsiveness and Distribution

When comparing ex vivo responsiveness of whole blood to stimulation with LPS following 1 months and 3 months of DPI, we found no significant alterations in TNFα, IL-6, or interleukin-1β (IL-1β) production (Fig. 1a, $n = 8$). Similarly, we found that PBMCs isolated from these volunteers did not demonstrate any changes in immune responsiveness following

ex vivo LPS stimulation (Fig. 1b, $n = 10$). In line with the cytokine levels, we did not measure any changes in specific immune cell populations or distributions. White blood cells, neutrophils, monocytes, and lymphocytes showed no meaningful changes in abundance across the different measurements (Fig. 1c, $n = 13$). Similarly, platelet counts also did not appear to be altered. This is in line with literature that shows low-dose aspirin does not affect platelet count in circulation but only affects their activation characteristics [14, 15].

DPI Does Not Change Markers of Systemic Inflammation in Patients with PAD

As CVD is driven by a state of chronic systemic inflammation, we sought to determine whether DPI has any effects on markers of systemic inflammation. We evaluated plasma samples from 33 patients with PAD who were enrolled in the DUAL-PAD study [10]. Mirroring the DUAL-CAD study, patients received 4 weeks of aspirin run-in followed by 3 months of DPI of low-dose aspirin and rivaroxaban. Circulating levels of inflammatory cytokines were than assessed for relative expression by Olink proteomics (Inflammation Target 96 panel). Principal component analysis (PCA) showed that baseline samples clustered closely with samples collected after 3 months of DPI (Fig. 2a). Similarly, paired statistical analysis comparing baseline with 3 months of DPI verified that there were no changes in systemic inflammation in these patients following combined treatment with low-dose aspirin and rivaroxaban (Fig. 2b).

DISCUSSION

Recent landmark studies such as the CANTOS [12] trial have convincingly demonstrated that inflammation plays a pivotal role in the pathophysiology of atherosclerotic diseases. However as a result of some practical restraints such as costs, these anti-inflammatory treatment avenues remain underutilized within the clinic. In the COMPASS trial, patients with severe forms of CAD and PAD benefited from DPI where low-dose aspirin is co-administered

Table 1 Patient characteristics

	DUAL-CAD ($n = 16$)	DUAL-PAD ($n = 33$)
Demographics		
Age (median, range)	72 [52–82]	67 [49–85]
Female sex (n, %)	5 (31.3)	11 (33.3)
Ethnicity (n, %)		
Caucasian	16 (100.0)	32 (97.0)
Other		1 (3.0)
Lifestyle		
Smoking behavior (n, %)		
Current	6 (37.5)	6 (18.2)
Former	9 (56.3)	25 (75.8)
Never	1 (6.3)	2 (6.1)
Vascular state		
Coronary episode leading to inclusion (n, %)		NA
Myocardial infarction	11 (68.8)	
Episode of unstable angina pectoris	4 (25.0)	
Stable angina pectoris	1 (6.3)	
PAD severity* leading to inclusion (n, %)	NA	
Intermittent claudication		27 (81.8)
CLTI		6 (18.2)
Previous intervention for PAD (n, %)	NA	24 (72.7)
Comorbidity		
Hypertension (n, %)	8 (50.0)	22 (66.7)
Hyperlipidemia (n, %)	7 (43.8)	14 (42.4)
Ischemic heart disease (n, %)	16 (100.0)	11 (33.3)
CVA/TIA (n, %)	3 (18.8)	4 (12.1)
PAD (n, %)	5 (31.3)	33 (100.0)

Table 1 continued

	DUAL-CAD (n = 16)	DUAL-PAD (n = 33)
Diabetes mellitus (n, %)	8 (50.0)	12 (36.4)
Drugs		
Lipid-lowering drugs (n, %)	14 (87.5)	33 (100.0)
Antihypertensive medication (n, %)	15 (93.8)	21 (63.6)
Physical examination		
BMI (median, range)	27.3 [21.1–38.6]	27.1 [21.0–38.2]
Blood pressure, mmHg (median, range)		
Systolic	130 [105–178]	148 [100–214]
Diastolic	76 [60–88]	76 [57–96]

Data are for 16 participants of the DUAL-CAD study who were evaluated for complete blood cell counts, whole blood stimulation, peripheral blood mononuclear cells (PBMC) isolation and stimulation, and cytokine levels of TNFα and IL-6; and patient characteristics of 33 participants of the DUAL-PAD study with inflammatory profiles determined using Olink proteomics

PAD peripheral arterial disease, *CAD* coronary artery disease, *CLTI* chronic limb-threatening ischemia, *CVA* cerebrovascular accident, *TIA* transient ischemic attack, *BMI* body mass index, *NA* not applicable

*Most severe presentation of PAD, now or in the past

alongside the factor X inhibitor rivaroxaban [3]. Given that there is considerable cross talk between platelets and the coagulation with the immune system [13], we aimed to determine whether any of the beneficial effects conveyed by DPI were due to dampening of inflammation.

Previously it was demonstrated that patients with active CAD not only have elevated levels of circulating cytokines but also elevated immune cell responsiveness to ex vivo LPS stimulation [11]. A strength of the current study design is that each participant serves as their own control, allowing for a comparison of DPI treatment to a background of aspirin monotherapy. This negates the need for an aspirin-only monotherapy control group which would require greater participant recruitment as well as appropriate randomization to control for variables. We did not observe any meaningful lowering of whole blood and/or PBMC ex vivo cytokine production following LPS stimulation from subjects with CAD. No changes in the distribution of immune cell populations were observed either. By accessing a larger cohort of 33 subjects with PAD and measuring relative levels of 96 inflammatory proteins in serum both at baseline and after 3 months of DPI, we did not measure a single significantly altered protein. Given that this proteomic analysis includes both pro-inflammatory as well as anti-inflammatory proteins, it is apparently clear that inflammation is neither changed by lowering of pro-inflammatory cytokines, nor by elevations in anti-inflammatory cytokines.

A limitation of this current study is the relatively short treatment period which may not be long enough to capture changes in systemic and cellular inflammation. However, in the COMPASS trial, the beneficial effects of DPI could be seen immediately upon switching medication. Therefore, given the lack of change in immune cell responsiveness, as well as changes in markers of systemic inflammation following 3 months of DPI, we do not believe that inflammation is playing a driving role in the beneficial mechanism of action of rivaroxaban combined with aspirin.

Given that DPI also failed to show meaningful changes in markers of macro- and microvascular endothelial function in the recently published DUAL-PAD study, we therefore hypothesize that the main mechanism by which DPI lowers the risk of severe outcomes is due to its primary role in reducing coagulation and thrombosis.

A further limitation of the current study is the relatively limited number of patients with CAD included. Despite this limitation, we do not observe any trends in ex vivo cytokine production or immune cell population shifts in the 13 subjects with CAD. Additionally, we feel

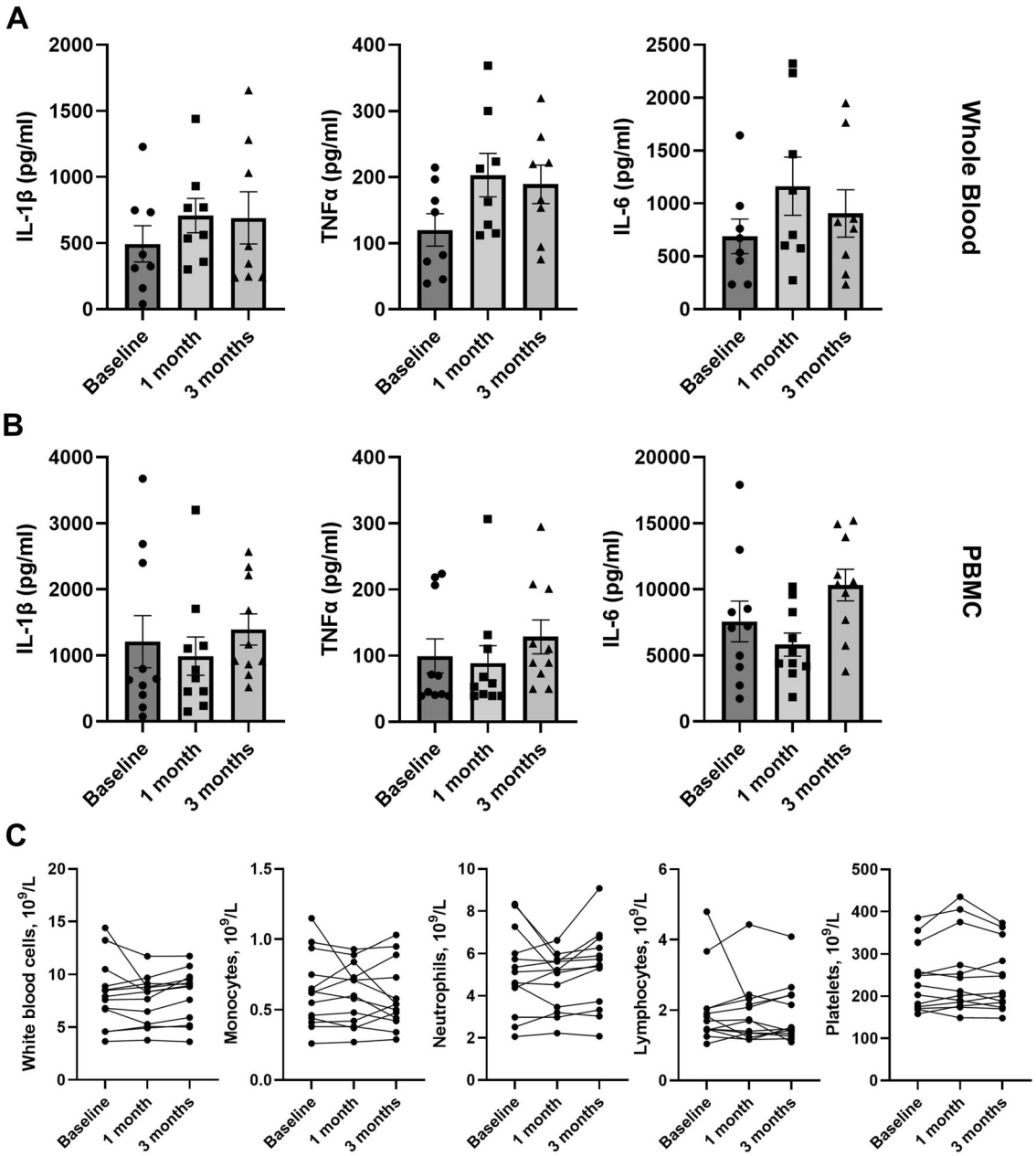

Fig. 1 Three months of dual-pathway inhibition (DPI) does not alter ex vivo immune cell responsiveness to lipopolysaccharide (LPS) stimulation nor circulating immune cell distribution. Levels of TNFα, IL-6, and IL-1β in ex vivo stimulated **a** whole blood (n = 8) and **b** peripheral blood mononuclear cells (PBMC) from study participants (n = 10). **c** Sysmex results showing the peripheral cell counts of white blood cells, monocytes, neutrophils, lymphocytes and platelets (n = 13). Data are represented as mean ± standard error of the mean (SEM), Wilcoxon signed-rank test

our conclusions are supported by the adequately sized proteomic analysis similarly showing no alterations in circulating inflammatory proteins in subjects with PAD after 3 months of DPI.

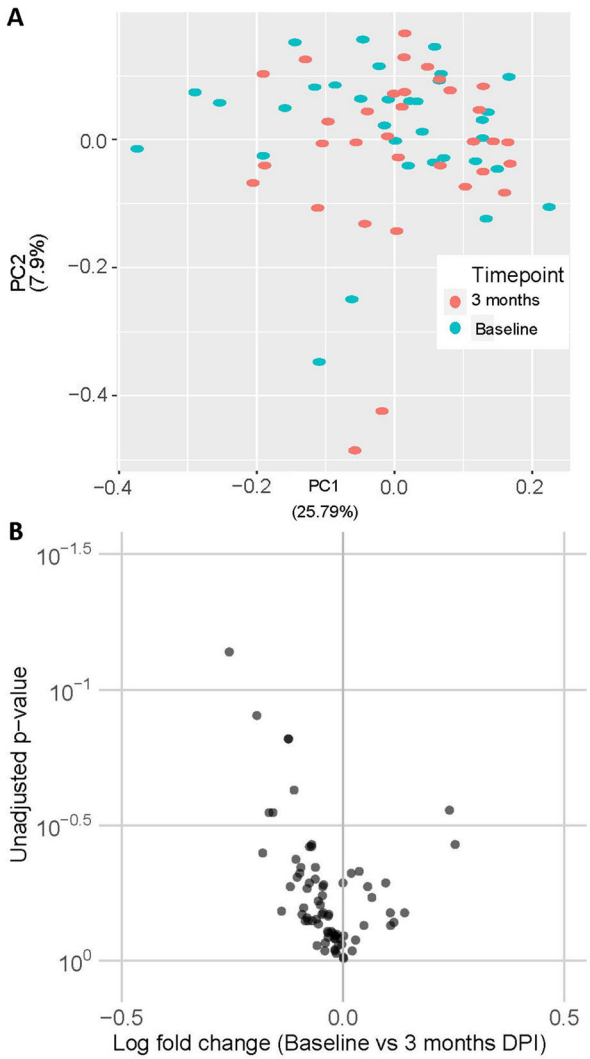

Fig. 2 Circulating markers of inflammation are not altered by 3 months of dual-pathway inhibition (DPI) in patients with peripheral artery disease (PAD). **a** Principal component analysis (PCA) comparing plasma levels of inflammatory proteins at baseline and after 3 months of DPI (n = 33, data dimensionality is visualized by principal component 1 (PC1) and PC2). **b** Volcano plot depicting a comparison of plasma levels of inflammatory proteins at baseline to 3 months of DPI in patients with PAD (n = 33)

CONCLUSION

Three months of low-dose aspirin combined with rivaroxaban treatment does not alter the immunophenotype of patients with CVD. DPI treatment did not alter relative levels of inflammatory proteins in the blood of patients with following 3 months of DPI. Additionally, 3 months of DPI did not alter immune cell responsiveness to ex vivo stimulation with LPS, nor the circulating distribution of different immune cell types. Therefore, our results indicate that the clinical benefit of DPI over aspirin alone [3] is more likely related to the intensified level of anticoagulation, and not to the inhibition of inflammatory pathways.

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Data Availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interest. Laszlo A. Groh, Loes H. Willems, Paula Fintelman, Michel M.P.J. Reijnen, Saloua El Messaoudi, and Michiel C. Warlé have nothing to disclose.

Ethical Approval. Approval of the Medical Research Ethics Committee Oost-Nederland (file number 2021-13291) and the local institutional review board was obtained. This study was conducted in accordance with the latest revision of the Helsinki Declaration of 1964 and Good Clinical Practice regulations and was registered at ClinicalTrials.gov on January 27, 2022 (registration number NCT05210725). Written informed consent was obtained from all participants.

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