Remote ischemic preconditioning (RIPC) protects against endothelial dysfunction in a human model of systemic inflammation. A randomized clinical trial

Marco Orlandi[1], Stefano Masi[2,3], Devina Bhowruth[2], Yago Leira[1,4,5], Georgios Georgiopoulos[6], Derek Yellon[7], Aroon Hingorani[8], Scott Chiesa[2], Derek J Hausenloy[7,9-12], John Deanfield[2], Francesco D'Aiuto[1]

1. Periodontology Unit, UCL Eastman Dental Institute and Hospital, University College London, London, United Kingdom
2. National Centre for Cardiovascular Prevention and Outcomes Institute of Cardiovascular Science, University College London, London, United Kingdom
3. Internal Medicine Unit, University of Pisa, Italy
4. Periodontology Unit, Faculty of Odontology, University of Santiago de Compostela & Medical-Surgical Dentistry Research Group, Health Research Institute of Santiago de Compostela, Santiago de Compostela, Spain
5. Clinical Neurosciences Research Laboratory, Health Research Institute of Santiago de Compostela, Santiago de Compostela, Spain
6. School of Biomedical Engineering and Imaging Sciences, King's College London, St Thomas Hospital, London, United Kingdom
7. The Hatter Cardiovascular Institute, University College London, London, United Kingdom
8. Institute of Cardiovascular Science, University College London, London, United Kingdom
9. Cardiovascular & Metabolic Disorders Program, Duke-National University of Singapore Medical School, Singapore

10. National Heart Research Institute Singapore, National Heart Centre, Singapore
11. Yong Loo Lin School of Medicine, National University Singapore, Singapore
12. Cardiovascular Research Center, College of Medical and Health Sciences, Asia University, Taiwan

RIPC and endothelial function

Corresponding author: Dr Marco Orlandi, Eastman Dental Institute, Rockefeller Building, 21 University Street, WC1E 6DE +447554421328 m.orlandi@ucl.ac.uk

Total word count: 6628

Basic Science Research, Inflammation, Endothelium/Vascular Type/Nitric Oxide, Ischemia, Vascular Biology

Total number of figures and tables: 5

Basic

Vascular Biology

ABSTRACT

Objective: Inflammation, oxidative stress and endothelial dysfunction are known to contribute to ischaemia-reperfusion (IR) injury. Remote Ischemic Preconditioning (RIPC) protects from endothelial dysfunction and the damage induced by IR. Using intensive periodontal treatment (IPT), an established human model of acute systemic inflammation, we investigated whether RIPC prevents endothelial dysfunction and modulates systemic levels of inflammation and oxidative stress.

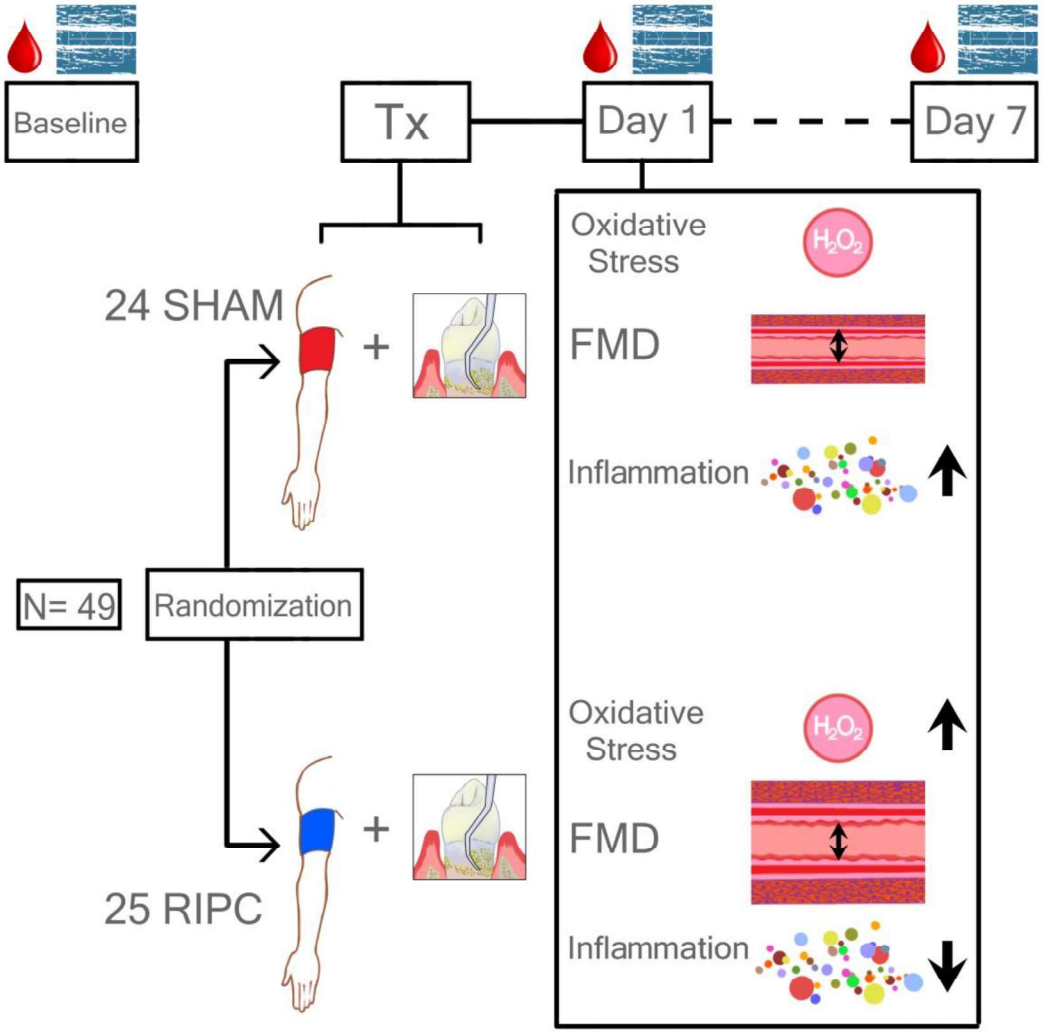
Approach and Results: Forty-nine participants with periodontitis (PD) were randomly allocated to receive either 3 cycles of IR on the upper limb (N 25, RIPC), or a sham procedure (N 24, Control) before IPT. Endothelial function assessed by flow-mediated dilatation (FMD) of the brachial artery, inflammatory cytokines, markers of vascular injury and oxidative stress were evaluated at baseline, Day 1 and Day 7 after IPT. Twenty-four hours post IPT, the RIPC group had lower levels of IL-10 and IL-12 compared to the Control group ($P<0.05$). RIPC attenuated the IPT-induced increase in IL-1β, E-selectin, sICAM3 and s-Thrombomodulin levels between the baseline and Day 1 (P for interaction<0.1). Conversely, oxidative stress was differentially increased at Day1 in the RIPC group compared to the Control group (P for interaction<0.1). This was accompanied by a better FMD (mean difference 1.75%, 95% CI 0.428 to 3.07, p=0.011). After 7 days from IPT, most of the inflammatory markers, endothelial dependent and independent vasodilation were similar between groups.

Conclusions: RIPC prevented acute endothelial dysfunction by modulation of inflammation and oxidation processes in patients with PD following exposure to an acute inflammatory stimulus.

Abstract Word Count:

Keywords: endothelium, periodontitis, inflammation, remote ischemic preconditioning

Clinical Trial Registration: NCT03072342 https://clinicaltrials.gov/ct2/show/NCT03072342



ABBREVIATIONS

RIPC	Remote Ischemic Preconditioning
IR	Ischemia-Reperfusion
IPT	Intensive Periodontal Treatment
PD	Periodontitis
FMD	Flow mediated dilatation
CVDs	Cardiovascular Diseases
PPD	Probing Pocket Depth
CAL	Clinical Attachment Level
REC	Recession
IL	Interleukin
IFN	Interferon
CRP	C-Reactive Protein
TNF	Tumor Necrosis Factor
sICAM-3	Soluble intercellular adhesion molecule-3
d-ROMs	Reactive oxygen metabolites
mtROS	Mitochondrial Reactive Oxygen Species
PBMC	Peripheral Blood Mononuclear Cells
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
GTN	Glyceryl trinitrate

GTNMD Glyceryl trinitrate mediated dilatation

INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of death and disability worldwide[1]. Central to the pathophysiology of many CVDs is endothelial dysfunction[2], and as such new treatment modalities are needed to prevent endothelial dysfunction and improve clinical outcomes in patients with CVD . In this regard, cycles of brief non-lethal ischemia and reperfusion to the arm or leg have been reported to prevent endothelial dysfunction induced by sustained ischemia and reperfusion, a phenomenon which has been termed remote ischemic preconditioning (RIPC)[3, 4].

The exact mechanisms by which RIPC protects the endothelium remain to be elucidated. However, modulation of systemic inflammation and oxidative stress have been implicated as possible contributing factors of the observed vasculo-protective effects[5-7]. Limited evidence, however is available on the interplay between RIPC, acute systemic inflammation, oxidative stress and endothelial function in humans.

Our group has developed and extensively characterised a novel model to study human inflammation: the intensive periodontal treatment model. Periodontitis is a common chronic inflammatory and infectious disease caused by a dysbiotic dental biofilm in susceptible individuals and affecting the tissues supporting the dentition[8]. Management of the disease relies upon professional cleaning of the teeth affected (mechanical disruption of the dental biofilm), resulting in a dramatic reduction of local gingival inflammation[9]. A single session of intensive periodontal treatment (IPT) not only improves gums' health but also

results in a one-week acute elevation of the systemic inflammation and oxidative stress[10]. This is thought to be related to the systemic dissemination of molecules of bacterial origin and a systemic inflammatory response associated with a profound but transient alterations of endothelial function[11].

Acute inflammation and its detrimental effects on the endothelium have been implicated as a plausible biological mechanism behind the link of common acute infections and increased vascular risk[12]. The exposure of endothelial cells to pro-inflammatory cytokines could stimulate the expression of tissue-factor, cell-surface adhesion molecules and induction of pro-coagulant activity.

Building on our previous experience, we designed a single-blind, parallel group, randomized controlled trial to evaluate whether RIPC can modulate the inflammatory and oxidative response and prevent endothelial dysfunction following an acute inflammatory stimulus induced by IPT in patients with PD.

METHODS

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

Population

Patients referred to the Eastman Dental Hospital, University College London (UK) for periodontal screening and therapy were invited to participate in this study if they had active generalized periodontitis defined as at least 30 periodontal pockets with probing pocket depth > 4mm and confirmed radiographic alveolar bone loss. Patients were excluded if they were: a) pregnant, breastfeeding or of childbearing potential, b) on chronic treatment (i.e.,

two weeks or more) with specific medications known to affect periodontal status (phenytoin or cyclosporine) within one month of the start of the study, c) suffering from any systemic disease (assessed by a medical history questionnaire), d) with limited mental capacity or language skills such that simple instructions cannot be followed or information regarding adverse events could not be provided, e) on any chronic medications or requiring antibiotic coverage for dental/periodontal procedures and f) had received a course of periodontal therapy in the preceding 6 months. All patients gave written informed consent. The study was approved by the London Queen Square Ethics Committee (06/Q0512/107).

Study Design

This was a randomized controlled clinical trial with a 7 day follow-up with two parallel groups. At baseline, full medical and dental histories were collected by a single trained examiner (MO). Anthropometric measures, including high, weight, waist and hip circumferences were recorded using standard protocols. Arterial blood pressure was measured in triplicate, and the average of the readings was recorded. Patients underwent full dental examinations, fasting blood samples collection and endothelial function assessment at baseline, as well as at 1 and 7 days following periodontal treatment (Figure 1). This trial was reported following the CONSORT guidelines[13] (Appendix 1).

Randomization

Following the baseline visit, study participants undergoing IPT were randomly assigned with the use of a computer-generated table and in a 1:1 ratio to receive RIPC (test group) or a sham procedure (control group) 30minutes before treatment. Smoking status, sex, age, and severity of periodontitis differences were accounted for in the randomization by

minimization[14]. Treatment assignments were concealed in opaque envelopes and revealed to the research staff performing the RIPC on the day the treatment was administered by the trial coordinator. The clinician performing IPT was unaware of the group allocation. The data were collected and analysed in masked fashion.

Periodontal examination and therapy

A single, trained dental examiner (MO) performed all dental assessments/treatment. The number of teeth, probing pocket depth (PPD), gingival recession (REC) and clinical attachment levels (CAL) were recorded. The presence or absence of supragingival dental plaque and gingival bleeding on probing on whole mouth was also recorded. All study participants underwent IPT within one month from the baseline visit. This consisted in a single-sitting full-mouth session of scaling and root planning, which was performed under local anaesthesia using hand and ultrasonic instruments as previously described[11].

Remote ischemic preconditioning (RIPC) and sham control procedure

RIPC was induced using a 9cm blood pressure cuff placed on the upper arm and inflated to a pressure of 200mmHg for 5 minutes followed by completed deflation for 5 minutes, a cycle which was repeated 3 times in total[15]. For the sham procedure (control group), a 9cm blood pressure cuff placed on the upper arm and inflated to a pressure of 10mmHg for 5 minutes followed by completed deflation for 5 minutes, a cycle which was repeated 3 times in total. IPT commenced after the completion of the RIPC protocol.

Vascular function

All the participants were instructed to fast for at least 8 hours, refrain from drinking beverages containing caffeine and to not smoke on the day of the examination. A

temperature controlled room (22 Celsius degrees) was used for all the vascular assessments. A high-resolution ultrasound machine (Acuson XP128 with a 7-MHz linear probe) for image acquisition and a semi-automatic edge detection software for post-acquisition analyses (Medical Imaging Applications, vascular research tools, version 5.6.7) was used to measure endothelium-dependent and independent vasodilatations of the brachial artery, as previously described[16]. After 10 minutes of rest, endothelium-independent dilatation was measured after sublingual administration of 25 μg of glyceryl trinitrate (GTN), according to the same recording protocol[16]. Brachial artery dilation was calculated as a percentage change from baseline to the peak diameters. A single examiner blinded to the RIPC or sham procedures, acquired all vascular data. Analysis of the FMD images was performed in a blinded fashion. The sonographer attended a training session (London Core Lab, London, UK) and completed a certification process which involved 10 repeat scans with < 2% variability in %FMD. All the patients were assessed at the same time (morning) of the day at each study visit (Baseline, Day1 and Day7).

Inflammatory and vascular biomarkers

At baseline, 24 hours and 7 days after IPT fasting blood samples were collected, immediately processed in several aliquots and stored at -70 degrees. Measures of a broad panel of inflammatory biomarkers was performed in blind fashion at the end of the trial using multiplex high sensitivity assays (Meso Scale Discovery, Maryland, USA) including interleukin-1β (IL-1β), IL-6, IL-8, IL-10, IL-12, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) according to manufacturer's instructions. Serum C-reactive protein (CRP) was measured by immunoturbidometry (Cobas, Roche Diagnostic, Mannheim, Germany). Soluble E-selectin, soluble P-selectin, soluble intercellular adhesion molecule-3 (sICAM-3)

and soluble thrombomodulin were assayed with a multiplex assay (Meso Scale Discovery, Maryland, USA). Coefficient of variation for all assays (intra and inter) were recorded and confirmed to be lower than 5%.

Oxidative stress

In this study, we chose a cumulative oxidative test in serum, reactive oxygen metabolites (d-ROMs) test, to estimate the total amount of oxidative metabolites of each sample. This test measures the serum concentration of hydro peroxides, a class of chemical oxidant species belonging to the wider group of reactive oxygen metabolites[17]. It has been previously used to measure total levels of circulating oxidative markers in patients undergoing periodontal treatment, showing reliable and reproducible results[18]. Further, mitochondria reactive oxygen species (mtROS) were measured at each study visit in peripheral blood mononuclear cells (PBMCs), isolated following standard procedures by density gradient centrifugation with Ficoll (Ficoll-Paque PLUS, GE, UK). Mitochondrial oxidative stress production was assessed by flow cytometry using the mitochondrial probes MitoSOX Red (Invitrogen, UK) as previously described[19].

Lipopolysaccharide (LPS) assay

Limulus Amebocyte Lysate was adopted for the detection of endotoxin (QCL-1000™, Lonza). According with this assay, Gram-negative bacterial endotoxin present in the samples catalyses the activation of a proenzyme in the LAL. The initial rate of activation was determined by the concentration of endotoxin present. This was measured photometrically

at 405-410 nm, after the reaction is stopped with stop reagent. The correlation between the absorbance and the endotoxin concentration was linear in the 0.1-1.0 EU/ml range. The concentration of endotoxin in a sample was calculated from the absorbance values of solutions containing known amounts of endotoxin standard.

Outcome assessment

The primary outcome of this study was the difference in brachial endothelial function assessed by FMD 24hrs following the dental treatment between study groups. Secondary outcomes included changes in the GTN-mediated dilatation (GTNMD), concentration of common inflammatory and circulating endothelial markers, d-ROMs, LPS and MitoSOX median fluorescent intensity (MFI) measured at 24hrs and at 7 days after IPT.

Statistical analysis

Based on our previous study[11], we estimated that a minimum of 22 per group patients should be enrolled into the study to detect a 2% difference in FMD between the test and control groups 24 hours after IPT (using a standard deviation of the mean difference of 1.6% at a two-sided alpha level of 5% and 90% power). Accounting for a potential dropout rate of 10%, a final sample size of 24 participants per group was recruited.

All is presented as mean values ± standard error or median and interquartile range (25^{th} to 75^{th} percentile). Categorical variables are shown as counts and percentages. Comparisons between groups (RIPC versus placebo) at each time point were based on the Independent samples Student's T test or the non-parametric Mann-Whitney test for continuous variables and the chi-squared test for categorical variables.

Differences in vascular and inflammatory markers within each group (RIPC or sham) and across study's duration (baseline, Day 1 and Day 7 after IPT) were initially assessed with the non-parametric Kruskal-Wallis test. To control the inflation of error rate in case of more than 2 comparisons among time points, we implemented the Dunn's test using rank sums with the Sidak adjustment. Remaining comparisons between groups with respect to main outcomes (FMD and GTNMD) and exposure variables (inflammatory and oxidative markers) were based on independent, pre-specified hypotheses. Therefore, no further correction for multiple comparisons was performed

Subsequently, linear mixed models with random effects (random intercept and random coefficient) and unstructured variance-covariance matrix were implemented to test the effect of RIPC versus the sham procedure on longitudinal changes in variables of interest across the acute (baseline to D1) and the sustained phase (baseline to D7) of the experiment. Demographic characteristics [i.e. age, sex, ethnicity, smoking and body mass index (BMI)] were pre-specified as covariates in the multivariable linear mixed models. An interaction term between exposure status and time (RIPC vs Sham*time) was used in the linear mixed model analysis to assess the differential effect of the randomly allocated intervention (RIPC versus placebo) on acute and prolonged changes in vascular and biochemical markers in comparison to placebo. For FMD specifically, we also tested linear mixed models involving additional independent variables with repeated measurements such as d-ROMs, hs-CRP, TNF-α and P-selectin to infer about potential temporal causality between longitudinal changes in endothelial function and in inflammatory/oxidative molecules. To ensure normal distribution of dependent variables, we performed inverse ranking normalization[20] prior to running the linear mixed models. Results of the linear mixed

model analysis are reported in the original scale for vascular markers to facilitate understanding the magnitude of the effect.

We used generalized structural equation modeling to fit multivariate linear mixed models with random intercept and random slope and test the overall interaction between RIPC and time (baseline to D1) on simultaneous repeated measurements in 4 key inflammatory variables (hs-CRP, TNF-α, IL-6 and INF-γ). We used the robust Huber/White/Sandwich estimator to derive the variance-covariance matrix of the estimates.

Finally, we aimed to disentangle the direct and indirect effects of the RIPC versus placebo on endothelial function and we employed structural equation models. In detail, we assessed the mediating effect of RIPC vs Control on FMD through P-selectin, CRP and TNF- and d-ROMs while controlling for the impact of age, sex, BMI and ethnicity. Standardized estimates were used in connecting paths. To address non-normality of variables and validate the indirect effects, we used Maximum Likelihood estimation with the robust estimator of the variance-covariance matrix (Huber/White/sandwich estimator). Standard errors and confidence intervals for the indirect effects were obtained through bootstrapping with 200 replicates. The comparative fit index (≥0.90 indicates acceptable fit) and the root mean square error of approximation (<0.06 indicates acceptable fit) were calculated to assess model fit for our main SEM. Statistical analysis was performed by STATA package, version 11.1 (StataCorp, College Station, Texas USA).

RESULTS

A total of 49 patients were recruited from April to October 2013 and randomly allocated to the control group (n = 25) and to the remote ischaemic conditioning group (n = 24) (Figure 1).

Study participants were middle-aged (46±9 years) with similar ethnicity and sex distribution, BMI, cardiovascular risk factors, levels of inflammatory cytokines, periodontal status, FMD and GTN-induced vasodilation (Table 1). No serious adverse events were recorded during the study. Table 2 reports changes in vascular parameters and circulating biomarkers across the study's follow up according to status of randomization (RIPC versus Control).

Vascular function

Changes in the endothelial function (primary outcome) from the baseline to the Day 1 visits were different in the RIPC (RIPC*baseline to Day 1 interaction=2.16%, 95% CI 1.45 to 2.88, p<0.001) (Figure 2A) compared to the control group (unadjusted between groups difference at Day 1 P=0.04 and adjusted difference=1.85%, 95% CI 0.463 to 3.24, p=0.009), suggesting that RIPC might attenuate the endothelial dysfunction induced by the acute inflammatory response following IPT. RIPC had similar impact on the changes of the GTNMD, so that GTN induced higher vasodilation in the RIPC (RIPC*baseline to Day 1 interaction=2.84%, 95% CI 1.13 to 4.55p=0.001) compared to the control group 24 hours post IPT (unadjusted between groups difference p=0.057 and adjusted difference 4.33%, 95% CI 1.08 to 7.57, p=0.009) (Figure 2B). These vascular changes recovered completely 7 days after IPT, when no differences between RIPC and control groups were observed for endothelial dependent and independent vasodilation (p>0.1 for both interaction terms and between-groups differences, Figure 2A, B). Furthermore, we observed a positive association between changes in FMD and GTNMD (coefficient=0.214%, 95% CI 0.133 to 0.295, P<0.001).

Markers of inflammation and vascular activation

RIPC attenuated the IPT-induced increase in IL-1β between the baseline and Day 1 (RIPC*baseline to Day 1=-1.57, 95% CI -1.90 to -1.23, p<0.001) and thus, the difference in IL-1β levels between groups (adjusted difference as compared to the sham procedure at Day 1= 0.195, 95% CI -0.937 to 1.33, p=0.735).

Among other inflammatory markers, in the RIPC group there was an attenuated rise in circulating E-selectin (P for interaction [baseline to Day 1]=0.056), sICAM3 (P for interaction [baseline to Day 1]=0.092 and P for interaction [baseline to Day 7]=0.078) and s-Thrombomodulin (P for interaction [baseline to Day 7]=0.06). IL-10 was increased at Day 1 in the placebo group as compared to the RIPC (mean adjusted difference=0.762, 95% CI 0.239 to 1.28, p=0.004) but no statistically significant interaction of treatment with changes in its levels was established. IL-12 was increased at Day 1 in the placebo group as compared to the RIPC (mean adjusted difference=0.692, 95% CI 0.129 to 1.26, p=0.016) but no statistically significant interaction of treatment with changes in this cytokine was established (P for interaction [baseline to Day 1]=0.376); in contrast, RIPC differentially increased the IL-12 levels at the end of the experiment (RIPC*baseline to Day 7=1.43, 95% CI 0.196 to 2.67, p=0.023). Importantly, by generalized structural equation modelling, we found a statistically significant overall interaction between type of allocated treatment before IPT and time (baseline to Day 1) on changes of the inflammatory array consisting of hs-CRP, TNF-α, IL-6 and INF-γ (p=0.01). This suggests that RIPC was able to attenuate the global systemic inflammatory response generated by the IPT compared to placebo.

No changes related to type of allocated treatment were observed for CRP, TNF-a, INF-γ, IL-6, IL-8 fluctuations across the pre-specified time points.

Oxidative stress

Participants who received RIPC exhibited higher increase in d-ROMs levels from baseline to Day 1 (RIPC*baseline to Day 1=0.835, 95% CI 0.255 to 1.42, p for interaction=0.005) and to Day 7 (RIPC*baseline to Day 7=0.768, 95% CI 0.185 to 1.35, p for interaction=0.01) when compared to the patients in the controls group. PBMC isolated from participants in the RIPC group revealed increased mtROS production compared to the those in the control group between baseline and Day 1 (RIPC*baseline to Day 1=0.666, 95% CI 0.018 to 1.314, p for interaction=0.044).

LPS

No substantial differences in plasma values of LPS in serum of patients in the RIPC and the control group were observed at all three time points (baseline, 24 hours and 7 days post IPT, p>0.1 for all). RIPC was not related with differential changes in LPS across the study for the two groups (p>0.1 for both interaction terms, baseline to 24hours and baseline to 7 days).

Interplay between inflammatory and oxidative markers

By linear mixed model analysis, we found an inverse linear association of changes in TNF-α (coefficient=-0.071, 95% CI -0.141 to -0.002, p=0.044), IL-8 (coefficient=-0.033, 95% CI -0.058/-0.008, p=0.01) and CRP (coefficient=-0.026, 95% CI -0.041 to -0.011, p=0.001) with fluctuations in FMD during the study's period after adjusting for age, sex, ethnicity, BMI and changes in SBP (Figure 3). When all 3 inflammatory markers were forced in the same model, CRP was the only biomarker to retain its association (coefficient=-0.019, 95% CI -0.035 to -0.002, p=0.028) with changes in FMD. We did not find evidence of concomitant changes in other circulating markers and fluctuations of endothelial function across the pre-specified

time points of the study (p>0.1 for all). Furthermore, changes in GTMD were inversely correlated with changes in TNF-α (coefficient=-0.575, 95% CI -.898/-.252, P<0.001), IFN-γ (coefficient=-0.116, 95% CI -.223 to -.0089, P=0.034), IL-12 (coefficient=-0.196, 95% CI -.348 to -.044, P=0.011], IL-6 (coefficient=-0.091, 95% CI -.167/-.0154, P=0.018), IL-8 (coefficient=-0.137, 95% CI -.239/ -.0345, P=0.009).

Mediation analysis

By structural equation models analysis, it was shown that while RIPC was a direct determinant of FMD changes (beta coefficient for the direct effect=1.67, 95% CI 0.342 to 2.999, p=0.014), no indirect effect through, hs-CRP, TNF-α and IL-8 (beta coefficient for the indirect effect=-0.31, 95% CI -0.061 to 0.123, p=0.510) was established.

DISCUSSION

This is the first clinical trial in humans showing that RIPC before a moderate inflammatory stimulus (induced by IPT) confers protection to the vasculature and that it was associated with modulation of markers of systemic inflammation and redox activity. The vascular benefits involved both endothelial dependent and independent vasodilation, suggesting a protective effect not limited to the endothelium but involving also the smooth muscle cells of the tunica media.

The ability of RIPC to modulate inflammatory responses has been previously investigated in humans with conflicting results. Some evidence reported that RIPC induces leukocyte inflammation gene expression[21], attenuates systemic neutrophil activation[3], and alters neutrophil function[22]. These changes occur within minutes after RIPC and are even more pronounced after 24 h.

By contrast, a lack of inflammatory modulation after endotoxemia following LPS administration has been described in a pilot experiment on healthy volunteers[23]. IPT represents a mixed infectious/inflammatory stimulus, as it induces a transient bacteraemia that is accompanied by an increase in the circulating levels of inflammatory cytokines. We previously documented modifications of the systemic endothelial function that track these changes of the inflammatory response[11]. We now show that, in the same model, RIPC is able to attenuate the rise of several inflammatory markers (including CRP, IL-1β and TNF-α) during the acute phase of the inflammatory response, supporting the hypothesis that RIPC could modulate systemic inflammation. Furthermore, we report that this effect is accompanied by a substantial attenuation of the endothelial dysfunction commonly recorded 24 hours after IPT. The potential protective effect of RIPC against the inflammatory stimulus generated by the IPT is confirmed by the reduced elevation of circulating P-selectin levels in the RIPC vs placebo groups. Importantly, through a formal mediation analysis we also show for the first time that, while there was a relationship between changes in FMD and the systemic levels of some inflammatory cytokines, inflammation is unlikely to mediate the impact of RIPC on the endothelial function. Similarly, the lack of substantial differences in LPS levels between groups at any time point during the study suggests that also the bacterial dissemination which follows the IPT is unlikely to account for its vascular effects. However, LPS does not reflect the levels of oral bacteria but could be subsequent to the far larger burden represented by the gut microbiome.

In this experiment we have also observed an improvement in GTNMD in the RIPC group. A deterioration in GTNMD following IPT was previously reported by our group[11]. Glyceryl trinitrate triggers vasodilation independently from the vascular endothelium and represents the vascular smooth muscle cell sensitivity to NO [24]. A deterioration in GTNMD might be the

result of underlying vascular smooth muscle dysfunction and possibly an impairment in ions ($Ca^{2+}$and K^{+})-mediated mechanisms regulating vascular smooth muscle cell contractility caused by the inflammatory response following IPT[25]. This finding warrants further investigation in further experiments.

We further investigated the impact of RIPC on PBMCs mitochondrial function assessed by superoxide production. PBMCs comprise a heterogeneous population of leukocytes including cells from the lymphoid system (predominantly T-cells, B-cells, and NK cells) and myeloid system (mainly monocytes). Although ROS are produced by several extracellular and intracellular processes, the mitochondria represent one of the main sources of oxidants. A role of mitochondria in the IR injury has been previously hypothesised, suggesting that RIPC might preserve cardiomyocyte mitochondrial function following IR[26]. Furthermore, we have recently reported that a lower PBMC mtROS production tracks the amelioration of FMD of the brachial artery observed 6 months following periodontal treatment[19]. These data suggested a potential role of mitochondria dysfunction in mediating the endothelial effects of both the IPT and RIPC. Unexpectedly, we found a higher mtROS in PBMCs of the RIPC compared to the placebo group 24 hours after IPT. The capacity of RIPC to induce a pro-oxidant environment is confirmed by the results of the dROMs test, showing that subjects receiving RIPC had a more substantial increase of dROMs than the control group 24 hours after IPT, and that this difference persisted a week later. It has been documented that an excessive generation of ROS and reactive nitrogen species within immune cells is linked to diminished inflammasome activation and a reduced inflammatory response[27]. Thus, we can speculate that an acute rise of mtROS production in inflammatory cells might impair their proinflammatory cytokine secretion and, through this mechanism, have a protective role on the endothelial function. This hypothesis could also explain the reduced cumulative

inflammatory response (hs-CRP, TNF-α, IL-6 and INF-γ) observed in the RIPC group 24 hours after the treatment. Other potential explanations of our results relates to the capacity of specific ROS to act as signalling mediators. dROMs are derivatives of reactive oxygen metabolites and their quantification indirectly estimates the total amount of hydroperoxides in serum representing an index of oxidant capacity. Hydrogen peroxide (H_2O_2) has been identified as a signalling mediator in the vasculature, having positive effect on the endothelium-dependent vasorelaxation[28]. Further, there is evidence to suggest a role of hydroperoxides in the endothelium-dependent vasodilation through COX-1-mediated release of PGE2. Finally, H_2O_2 acts directly on smooth muscle by hyperpolarization through KCa channel activation leading to relaxation[29]. This would also explain the improved GTN-induced vasorelaxation at 24 hours after IPT observed in the RIPC compared to the placebo group. The importance of an adequate ROS response to the RIPC is confirmed by the loss of preconditioning protection when cardiomyocytes are treated with antioxidants[30, 31]. Although these findings might provide potential explanations to our results, the unexpected nature of our results, the lack of data describing the changes of PBMC mtROS production and its association with endothelial activation/protection after an acute inflammatory stimulus impose a careful interpretation of our results and confirmation in future investigations.

Our study has some limitations. The anti-inflammatory effects of RIPC observed may be specific to the IPT model and their relevance in other human models of acute inflammation remains to be tested. Although we included healthy participants with no other systemic conditions known to impact on the endothelium (such as hypertension, heart failure, atherosclerosis, hypercholesterolemia, diabetes mellitus), we cannot rule out an alternative mechanism of protection of RIPC on vascular dysfunction A difference in the levels of dROM

at the baseline visit, could confound the interpretation of the results, although the overproduction of ROS following RIPC is confirmed by the data on the mtROS. On the other hand, our study has several strengths. The randomized design and masked assessment contribute to high internal validity. Our group has extensively characterised the inflammatory and oxidative stress responses to IPT, making this a solid model to study the complex interaction between inflammation and endothelial function in humans. The presence of a single blind vascular examiner reduced the variability of our vascular measures. Finally, data on a wide range of potential mediators of the benefits related to the RIPC on the endothelial function were acquired and analyzed using a robust statistical methodology to ascertain the potential influence of many parameters on the link between RIPC, inflammation and vascular phenotype.

RIPC performed before an acute inflammatory stimulus can modulate both acute inflammation and endothelial cell activation. This resulted in an improvement of endothelial function and was associated with a transient increase in oxidative stress. A wide range of infective disorders and iatrogenic procedures can cause severe systemic inflammation. Acute systemic inflammation is associated with an increase in the risk of cardiovascular events that may persist for days or weeks. The present study demonstrates that RIPC may be tested as prevention technique to protect the endothelium from the negative impact of an acute inflammatory response. Further research is required to confirm the protective role of RIPC against the potential adverse effects of inflammation.

ACKNOWLEDGMENTS

We would like to thank Dr Jeanie Suvan, Ms Banbai Hirani, Ms Tiff Mellor, Ms Chiara Curra and Ms Kasia Niziolek for their invaluable assistance and the Eastman Dental Hospital for the valuable support in the recruitment.

SOURCE OF FUNDING

This study was funded thanks to a UCL Impact Award to Marco Orlandi partially supported with a fellowship grant by Johnson and Johnson Consumer Services EAME Limited and a fast-track grant to Francesco D'Aiuto from the UCL Biomedical Research Centre (CRDC - F189) and it was performed at UCL/UCLH which receives support from the UCL Biomedical Research Centre who obtained funding from the NIHR. Marco Orlandi holds a NIHR Clinical Lectureship awarded by the National Institute for Health Research (NIHR). Yago Leira holds a research contract with Fundación Instituto de Investigación Sanitaria de Santiago de Compostela (fIDIS) and a Senior Clinical Research Fellowship supported by the UCL Biomedical Research Centre who receives funding from the NIHR (NIHR-INF-0387). Derek Hausenloy was supported by the British Heart Foundation (CS/14/3/31002), the Duke-NUS Signature Research Programme funded by the Ministry of Health, Singapore Ministry of Health's National Medical Research Council under its Clinician Scientist-Senior Investigator scheme (NMRC/CSA-SI/0011/2017), Centre Grant, and Collaborative Centre Grant scheme (NMRC/CGAug16C006). Scott Chiesa holds a British Heart Foundation project grants (PG/18/45/33814 and PG/19/31/34343). Georgios Georgiopoulos was supported by a postdoctoral research fellowship from the Alexander S. Onassis Foundation.

DISCLOSURES

None

1

2

3

4

5

6

REFERENCES

1. Group WCRCW. World health organization cardiovascular disease risk charts: Revised models to estimate risk in 21 global regions. *Lancet Glob Health*. 2019;7:e1332-e1345
2. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: Testing and clinical relevance. *Circulation*. 2007;115:1285-1295
3. Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, Vallance P, Deanfield J, MacAllister R. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. *Circulation*. 2001;103:1624-1630
4. Loukogeorgakis SP, Panagiotidou AT, Broadhead MW, Donald A, Deanfield JE, MacAllister RJ. Remote ischemic preconditioning provides early and late protection against endothelial ischemia-reperfusion injury in humans: Role of the autonomic nervous system. *J Am Coll Cardiol*. 2005;46:450-456
5. Chen G, Thakkar M, Robinson C, Dore S. Limb remote ischemic conditioning: Mechanisms, anesthetics, and the potential for expanding therapeutic options. *Frontiers in neurology*. 2018;9:40
6. Halestrap AP, Clarke SJ, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta*. 2007;1767:1007-1031
7. Das M, Das DK. Molecular mechanism of preconditioning. *IUBMB life*. 2008;60:199-203
8. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet*. 2005;366:1809-1820
9. Suvan J, Leira Y, Moreno Sancho FM, Graziani F, Derks J, Tomasi C. Subgingival instrumentation for treatment of periodontitis. A systematic review. *J Clin Periodontol*. 2020;47 Suppl 22:155-175
10. D'Aiuto F, Parkar M, Tonetti MS. Periodontal therapy: A novel acute inflammatory model. *Inflamm Res*. 2005;54:412-414
11. Tonetti MS, D'Aiuto F, Nibali L, Donald A, Storry C, Parkar M, Suvan J, Hingorani AD, Vallance P, Deanfield J. Treatment of periodontitis and endothelial function. *N Engl J Med*. 2007;356:911-920
12. Teles R, Wang CY. Mechanisms involved in the association between periodontal diseases and cardiovascular disease. *Oral diseases*. 2011;17:450-461
13. Schulz KF, Altman DG, Moher D. Consort 2010 statement: Updated guidelines for reporting parallel group randomised trials. *Bmj*. 2010;340:c332
14. Scott NW, McPherson GC, Ramsay CR, Campbell MK. The method of minimization for allocation to clinical trials. A review. *Control Clin Trials*. 2002;23:662-674
15. Loukogeorgakis SP, Williams R, Panagiotidou AT, Kolvekar SK, Donald A, Cole TJ, Yellon DM, Deanfield JE, MacAllister RJ. Transient limb ischemia induces remote preconditioning and remote postconditioning in humans by a k(atp)-channel dependent mechanism. *Circulation*. 2007;116:1386-1395
16. Charakida M, Masi S, Luscher TF, Kastelein JJ, Deanfield JE. Assessment of atherosclerosis: The role of flow-mediated dilatation. *European heart journal*. 2010;31:2854-2861
17. Colombini F, Carratelli M, Alberti A. Oxidative stress, d-roms test, and ceruloplasmin. *Free Radic Res*. 2016;50:447-453

18. D'Aiuto F, Nibali L, Parkar M, Patel K, Suvan J, Donos N. Oxidative stress, systemic inflammation, and severe periodontitis. *J Dent Res*. 2010;89:1241-1246
19. Masi S, Orlandi M, Parkar M, Bhowruth D, Kingston I, O'Rourke C, Virdis A, Hingorani A, Hurel SJ, Donos N, D'Aiuto F, Deanfield J. Mitochondrial oxidative stress, endothelial function and metabolic control in patients with type ii diabetes and periodontitis: A randomised controlled clinical trial. *Int J Cardiol*. 2018;271:263-268
20. Stamatelopoulos K, Georgiopoulos G, Athanasouli F, Nikolaou PE, Lykka M, Roussou M, Gavriatopoulou M, Laina A, Trakada G, Charakida M, Delialis D, Petropoulos I, Pamboukas C, Manios E, Karakitsou M, Papamichael C, Gatsiou A, Lambrinoudaki I, Terpos E, Stellos K, Andreadou I, Dimopoulos MA, Kastritis E. Reactive vasodilation predicts mortality in primary systemic light-chain amyloidosis. *Circ Res*. 2019;125:744-758
21. Konstantinov IE, Arab S, Kharbanda RK, Li J, Cheung MM, Cherepanov V, Downey GP, Liu PP, Cukerman E, Coles JG, Redington AN. The remote ischemic preconditioning stimulus modifies inflammatory gene expression in humans. *Physiol Genomics*. 2004;19:143-150
22. Shimizu M, Saxena P, Konstantinov IE, Cherepanov V, Cheung MM, Wearden P, Zhangdong H, Schmidt M, Downey GP, Redington AN. Remote ischemic preconditioning decreases adhesion and selectively modifies functional responses of human neutrophils. *J Surg Res*. 2010;158:155-161
23. Zwaag J, Beunders R, Warlé MC, Kellum JA, Riksen NP, Pickkers P, Kox M. Remote ischaemic preconditioning does not modulate the systemic inflammatory response or renal tubular stress biomarkers after endotoxaemia in healthy human volunteers: A single-centre, mechanistic, randomised controlled trial. *Br J Anaesth*. 2019;123:177-185
24. Maruhashi T, Soga J, Fujimura N, Idei N, Mikami S, Iwamoto Y, Kajikawa M, Matsumoto T, Hidaka T, Kihara Y, Chayama K, Noma K, Nakashima A, Goto C, Higashi Y. Nitroglycerine-induced vasodilation for assessment of vascular function: A comparison with flow-mediated vasodilation. *Arterioscler Thromb Vasc Biol*. 2013;33:1401-1408
25. Ohkawa F, Ikeda U, Kanbe T, Kawasaki K, Shimada K. Effects of inflammatory cytokines on vascular tone. *Cardiovasc Res*. 1995;30:711-715
26. Kuznetsov AV, Javadov S, Margreiter R, Grimm M, Hagenbuchner J, Ausserlechner MJ. The role of mitochondria in the mechanisms of cardiac ischemia-reperfusion injury. *Antioxidants (Basel)*. 2019;8:454
27. Mao K, Chen S, Chen M, Ma Y, Wang Y, Huang B, He Z, Zeng Y, Hu Y, Sun S, Li J, Wu X, Wang X, Strober W, Chen C, Meng G, Sun B. Nitric oxide suppresses nlrp3 inflammasome activation and protects against lps-induced septic shock. *Cell Res*. 2013;23:201-212
28. Bretón-Romero R, Lamas S. Hydrogen peroxide signaling in vascular endothelial cells. *Redox Biol*. 2014;2:529-534
29. Thengchaisri N, Kuo L. Hydrogen peroxide induces endothelium-dependent and -independent coronary arteriolar dilation: Role of cyclooxygenase and potassium channels. *Am J Physiol Heart Circ Physiol*. 2003;285:H2255-2263
30. Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem*. 1998;273:18092-18098

31. Vanden Hoek T, Becker LB, Shao ZH, Li CQ, Schumacker PT. Preconditioning in cardiomyocytes protects by attenuating oxidant stress at reperfusion. *Circ Res*. 2000;86:541-548

HIGHLIGHTS

- Remote Ischemic Preconditioning (RIPC) has been investigated as method to attenuate the ischemia reperfusion damage that follows an acute ischemic injury.
- This mechanistic trial tested the effect of RIPC on endothelial function in a human model of systemic inflammation.
- These results suggest a protective effect of RIPC on endothelial function via the modulation of the inflammatory response and the redox activity following exposure to a validated systemic acute inflammatory stimulus in humans.

FIGURE LEGENDS

Figure 1 CONSORT Study Flowchart

Figure 2a Mean flow-mediated dilatation during the study duration

I bars represent standard error (SE). Data are for the 23 patients in the test group and the 24 patients in the control-treatment group. FMD % changes. Please notes values are adjusted for age, sex, smoking, body weight, and ethnicity. P values for the interaction terms Baseline to 24hrs (P<0.001). P-values are derived from linear mixed model analysis

Figure 2b Mean GTN-mediated dilatation during the study duration

I bars represent standard error (SE). Data are for the 23 patients in the test group and the 24 patients in the control-treatment group. GTN % changes. Please notes values are adjusted for age, sex, smoking, body weight, and ethnicity. P values for the interaction terms Baseline to 24hrs (P=0.007). P-values are derived from linear mixed model analysis

Figure 3 Changes in hs- CRP and fluctuations in FMD during the study duration. Please notes values are adjusted for age, sex, ethnicity, BMI and changes in SBP.

Table 1. Baseline charactheristics of the 2 study groups

	Control (N=24)	RIPC (N=23)
Age (years)	47±9	45±9
Sex, Male (%)	14(56.0%)	11(45.8%)
Ethnicity, Caucasian (%)	15(60.0%)	15(62.5%)
Body Mass Index (Kg/m^2)	26.5±3.8	26.1±3.7
Waist circumference (cm)	92±8	93±8
Hip circumference (cm)	106±8	105±6
Smoking, current (%)	7 (28.0%)	8 (33.3%)
SBP (mmHg)	120±16	118±10
DBP (mmHg)	77.58±8.51	76.43±8.08
TC (mmol/L)	5.18±0.81	5.17±0.78
TG (mmol/L)	1.11±0.53	1.06±0.26
hs-CRP (mg/L)	2.05±2.19	1.74±1.72
IL-1β (pg/ml)	0.35±0.2	0.21±0.17
IL-6 (pg/ml)	1.15 (0.63-1.79)	0.92(0.38-2.00)
IL-8 (pg/ml)	11.22±3.45	11.64±4.47
IL-10 (pg/ml)	6.26±8.01	4.08±3.82
IL-12 (pg/ml)	0.66±0.64	0.74±0.48
TNF-α, (pg/ml)	4.00±1.92	3.52±1.85
INF-γ, (pg/ml)	2.39±2.46	3.15±3.15
sE-Selectin (pg/ml)	21.26±16.43	20.75±15.20
sP-Selectin (pg/ml)	122.8962.12	114.92±43.91

s-ICAM3 (pg/ml)	1.92±3.86	2.02±3.39
s-TM (pg/ml)	5.30±3.98	5.47±6.06
d-ROMs (Carr/U)	455.04±89.17	383.60±98.05
PBMC mtROS (MitoSOX, MFI)	26.63±11.54	24.05±10
LPS (EU)	0.88±0.59	1.09±0.91
FMD (%)	6.28±2.56	6.28±3.68
GTNMD (%)	17.40±7.14	19.52±7.64
IPT Time (minutes)	144±25	128±24
PPD (cm)	4.16±.82	3.84±.56
REC (cm)	.85±.86	.86±.82
NPKTS (n)	69.33±28.96	61.00±21.81
FMPS (%)	63.97±16.23	58.90±15.6
FMBS (%)	49.86±21.97	50.05±16.04
NTEETH (n)	28.46±2.72	28.57±2.84

Values are expressed in Mean±SD for continuous variables and number (%) for categorical variables.

SBP systolic blood pressure, **DBP** diastolic blood pressure**, TC** total cholesterol, **TG** Triglycerides, **hs-CRP** high sensitivity C-reactive protein, **IL-1β** Interleukin-1β, **IL-6** Interleukin-6, **IL-8** Interleukin-8, **IL-10** Interleukin-10, **IL-12** Interleukin-12, **TNF-α** Tumor Necrosis Factor-α, **INF-γ** Interferon-γ, **s-TM** soluble Thrombomodulin, **d-ROMs** reactive oxygen metabolites, **mtROS** Mitochondrial Reactive Oxygen Species, **LPS** Lipopolysaccharides, **FMD** Flow Medicated Dilation**, GTNMD** glyceryl trinitrate mediated dilatation**, IPT** Intensive Periodontal Therapy; **PPD** gingival probing pocket depth, **REC**

gingival recessions, **NPKTS** number of periodontal lesions with probing pocket depth greater than 4mm, **FMPS** full mouth dental plaque score, **FMBS** full mouth gingival bleeding score, **NTEETH** number of teeth.

TABLE 2. Changes in vascular indices and circulating biomarkers across the study's follow up according to status of randomization (RIPC versus Control)

Treatment	Variable	Baseline	Day 1	**Day 7**	***p* value**
Control	FMD	6.15 (4.21-8.34)	**2.94 (1.95-5.06)*,†**	4.78 (4.12-6.75)	<0.001
RIPC		5.03 (3.75-7.9)	3.89 (2.9-7.93)	5.02 (3.66-8.55)	0.471
Control	GTNMD	16.9 (12.3-22.6)	14.2 (9.84-18.8)	13.8 (12.2-19)	0.309
RIPC		17.8 (13.8-25.5)	18 (14.8-24.8)	17.6 (14.8-23.8)	0.971
Control	TNF-α	3.47 (2.45-5.46)	4.47 (3.47-7.74)	5.28 (3.52-6.27)	0.121
RIPC		3.16 (1.94-4.75)	3.86 (2.28-5.16)	3.67 (3.14-5)	0.29
Control	hs-CRP	1.30 (0.9-2.7)	**8 (4.5-14.2)***	2.15 (1.45-4.55)	<0.001
RIPC		1.00 (0.6-2.8)	**5.25 (3.3-8.5)***	1.85 (1.1-2.8)	<0.001
Control	IFN-γ	1.42 (0.50-4.44)	**10.8 (2.54-26.7)***	2.83 (1.43-7.13)	0.012
RIPC		2.57 (0.66-4.68)	4.59 (1.88-11.1)	2.33 (1.33-3.48)	0.174
Control	IL-1β	0.36 (0.21-0.50)	0.45 (0.22-0.76)	0.29 (0.11-0.51)	0.557
RIPC		0.25(0.02-0.36)	1.27 (0.187-3.17)	0.208 (0.18-0.66)	0.661
Control	IL-6	1.15 (0.63-1.79)	**4.05 (2.2-7.19)***	1.56 (1.17-	<0.001

				2.99)	
RIPC		0.92 (0.38-2)	**3.9 (1.95-5.43)***	1.43 (1.13-2.69)	<0.001
Control	IL-8	11.4 (8.04-13.9)	13.3 (7.86-17.6)	13.8 (7.89-18.6)	0.366
RIPC		11.7 (8.9-16.3)	9.43 (7.67-14.9)	11.9 (7.7-6.0)	0.614
Control	IL-10	3.8 (1.92-6.61)	**7.49 (4.92-11.6)*,†**	4.58 (3.4-9.17)	0.064
RIPC		2.77 (1.25-6.75)	3.86 (2.1-6.94)	4.67 (1.85-8.26)	0.532
Control	IL-12	0.38 (0.19-0.83)	**2.13 (1.11-4.2)*,†**	**0.55 (0.26-1.31)†**	0.004
RIPC		0.90 (0.46-1.03)	1.11 (0.57-1.61)	2.17 (1.13-3.22)*	0.061
Control	s-ICAM3	1.14 (0.79-1.54)	1.05 (0.91-1.57)	1.28 (0.85-1.65)	0.793
RIPC		1.39 (0.83-1.78)	1.22 (0.93-1.49)	1.3 (1.14-1.51)	0.707
Control	sE-Selectin	17 (11.7-22.9)	20.2 (15- 24.8)	17.5 (10.8-21.9)	0.459
RIPC		15.9 (10.7-24.9)	18.7 (10.7-22.5)	14.4 (10.5-21.2)	0.552
Control	sP-Selectin	102 (85.1-142)	103 (64.3-148)	125 (76.6-141)	0.794
RIPC		102 (83.9-155)	80.6 (68-106)	91.1 (69.7-128)	0.163

Control	s-TM	4.7 (3.2-5.79)	4.31 (3.45-5.9)	4.5 (3.45-5.27)	0.957
RIPC		4.15 (3.49-4.82)	4 (3.24-4.62)	3.57 (3.38-4.44)	0.383
Control	dROMs	451 (409-493)†	470 (395-529)	436 (388-491)	0.804
RIPC		388 (326-439)	443 (393-525)	467 (366-544)*	0.038
Control	PBMC mtROS	23.1 (19.1-28.6)	23 (17-31.5)	23.3 (17.6-26.7)	0.774
RIPC		23.1 (17.2-25.3)	25.6 (20.1-34.2)	21.7 (16.5-34.3)	0.353
Control	Lymphocytes mtROS	19.6 (13.8-25.3)	18.5 (14.4-23.7)	18.9 (14.2-22.5)	0.966
RIPC		17.6 (12.7-23.9)	18.6 (14.1-33.8)	17.5 (13.2-30.5)	0.556
Control	Monocytes mtROS	55.2 (33.7-100)	87.8 (37.3-140)	56.2 (41.8-91.4)	0.342
RIPC		44.6 (25.9-82)	83.5 (42-167)*	50 (30.8- 108)	0.067

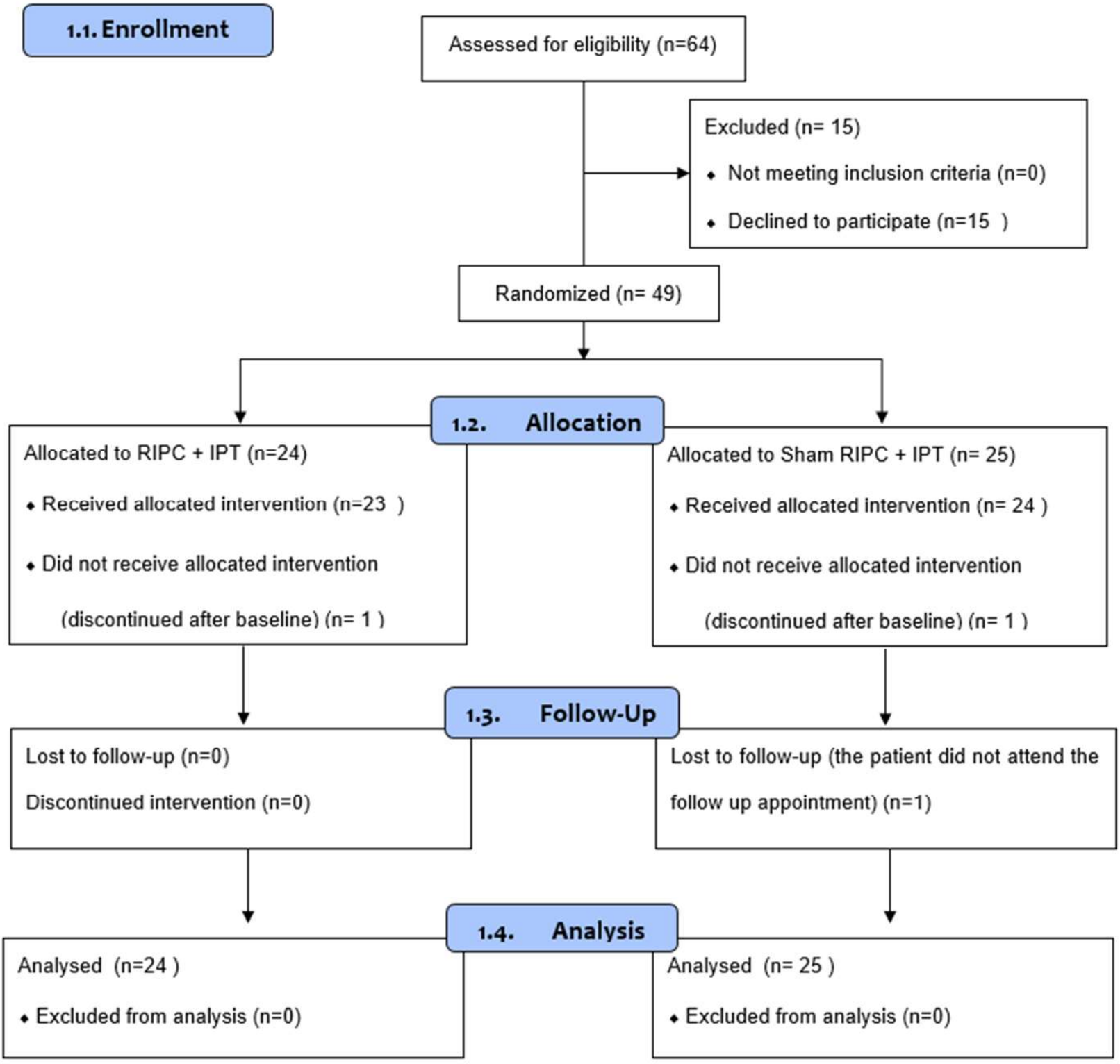
*** indicates statistically significant within-group difference from the reference category (baseline) after Sidak adjustment for multiple comparisons**

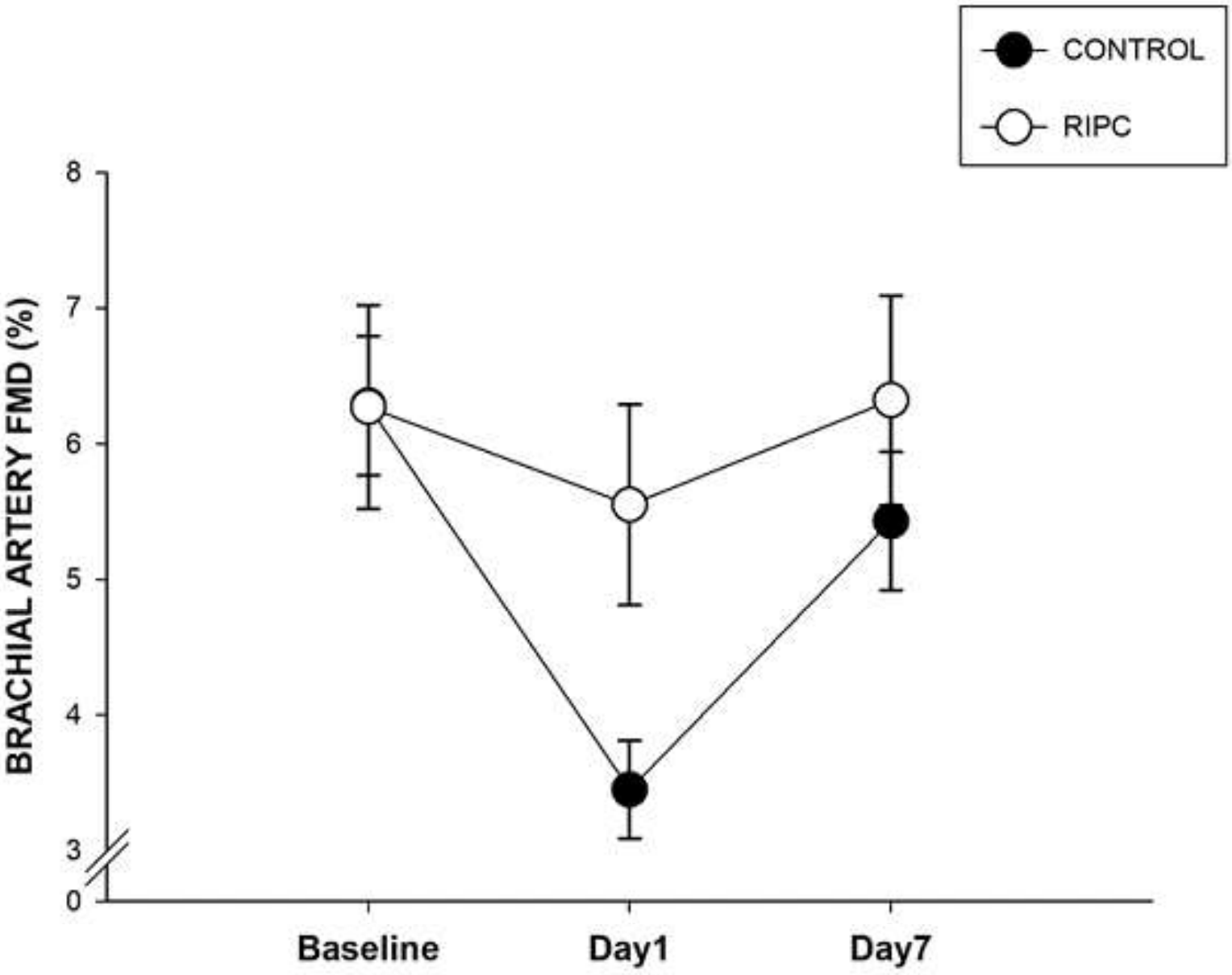
† indicates statistically significant between groups difference for the same time point (baseline,24 hours or Day 7)

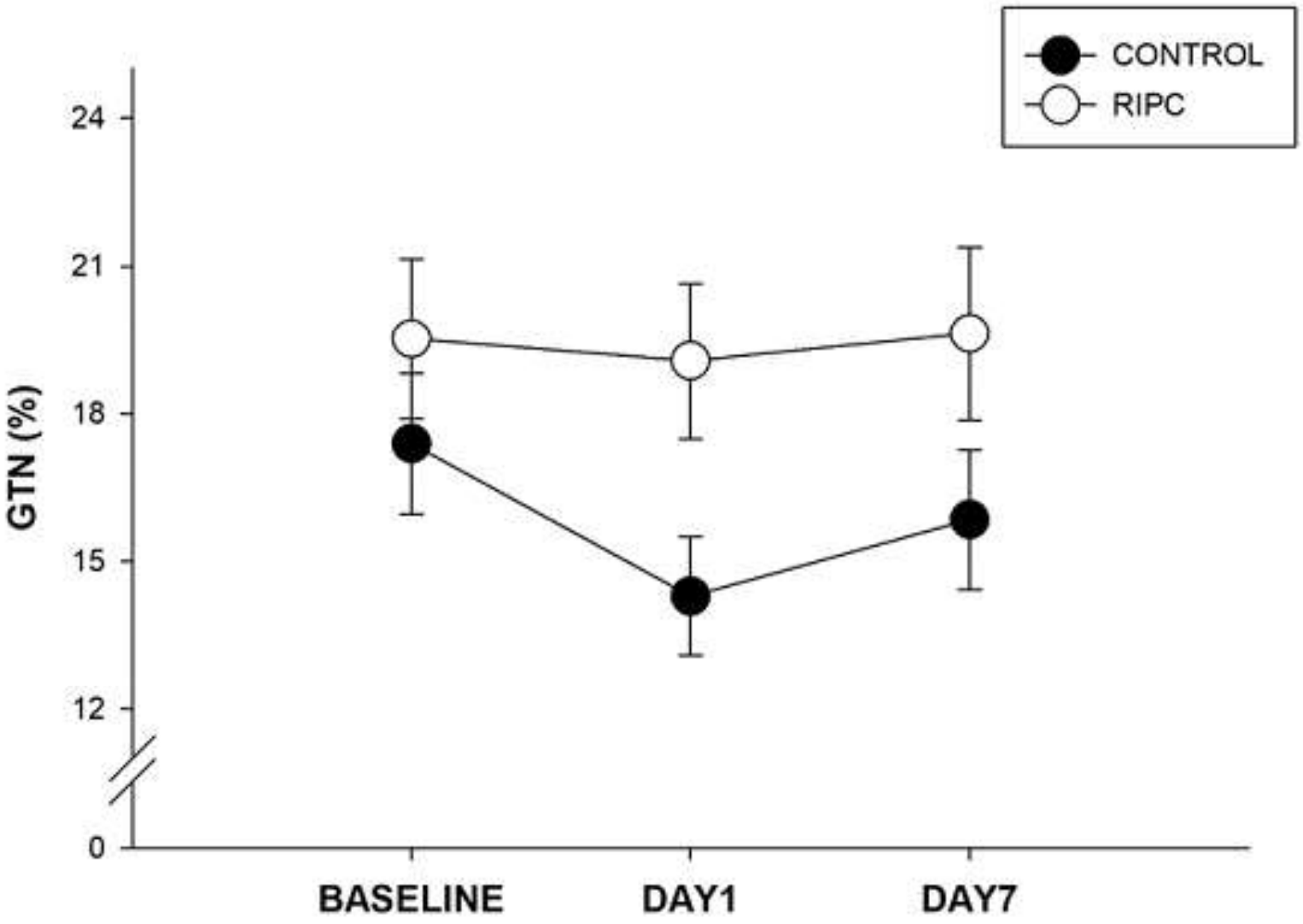
Values are expressed in Mean (CI)

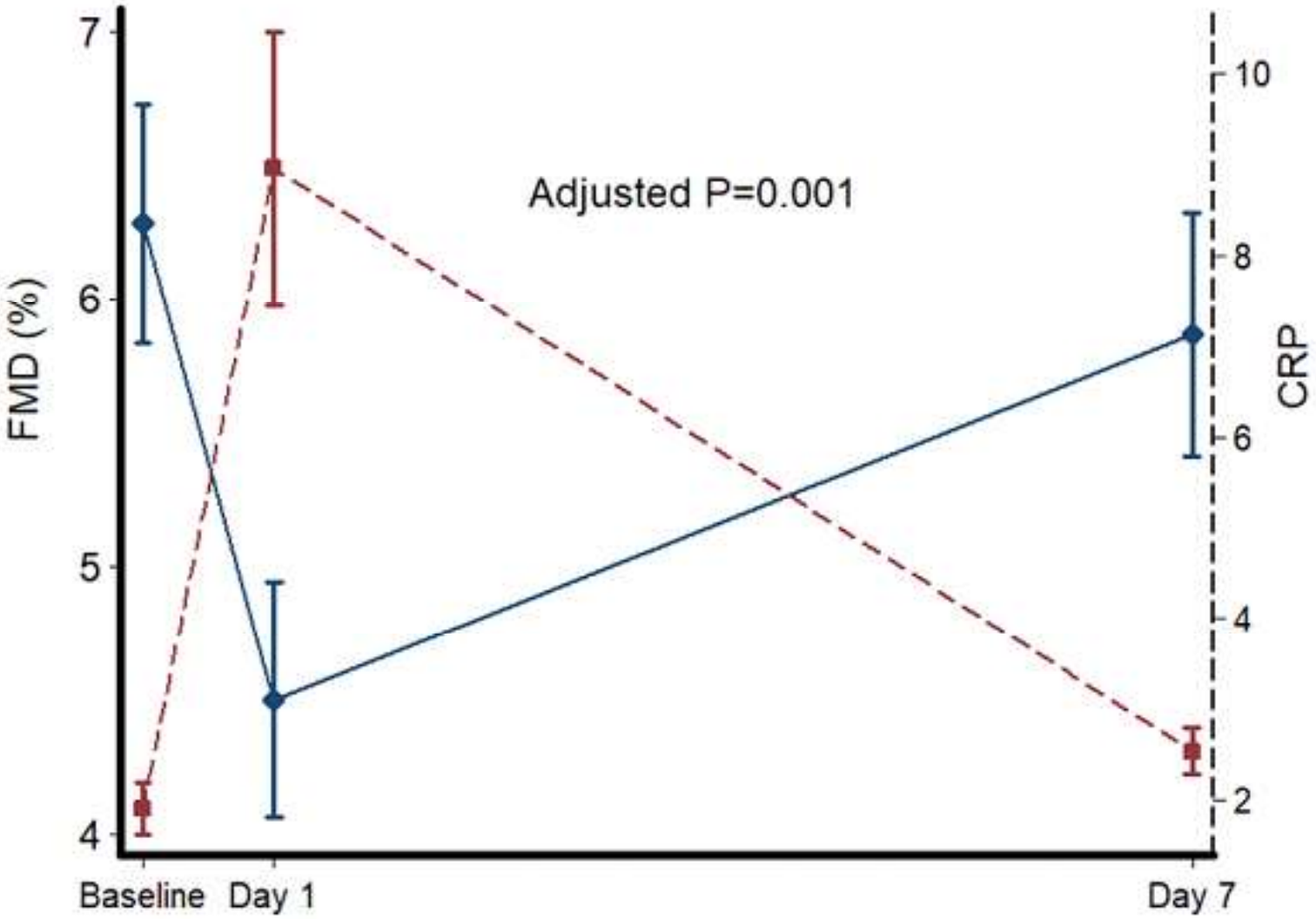
hs-CRP high sensitivity C-reactive protein, **IL-1β** Interleukin-1β, **IL-6** Interleukin-6, **IL-8** Interleukin-8, **IL-10** Interleukin-10, **IL-12** Interleukin-12, **TNF-α** Tumor Necrosis Factor-α, **INF-γ** Interferon-γ, **s-TM** soluble Thrombomodulin, **d-ROMs** reactive oxygen metabolites, **mtROS** Mitochondrial Reactive Oxygen Species, **LPS** Lipopolysaccharides, **FMD** Flow Medicated Dilation**, GTNMD** glyceryl trinitrate mediated dilatation**,**

CONSORT 2010 Flow Diagram




CONTROL
RIPC
BRACHIAL ARTERY FMD (%)
8
7
6
5
4
3
0
Baseline
Day1
Day7


CONTROL
RIPC
24
21
18
15
12
0
GTN (%)
BASELINE
DAY1
DAY7


7
6
5
4
FMD (%)
10
8
6
4
2
CRP
Adjusted P=0.001
Baseline
Day 1
Day 7