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Sixteen-Week physical activity intervention in subjects with increased cardiometabolic risk shifts innate immune function towards a less pro-inflammatory state.

A 16-week physical activity intervention in subjects with increased cardiometabolic risk shifts innate immune function towards a less pro-inflammatory state

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ABSTRACT

Background. Low-grade inflammation, largely mediated by monocyte-derived macrophages, contributes to atherosclerosis. Sedentary behavior (SB) is associated with atherosclerosis and cardiovascular (CV) diseases. We examined whether reducing SB and improving walking time improves monocyte inflammatory phenotype in subjects with increased CV risk.

Methods and Results. Across two waves, sixteen individuals with increased CV risk performed a 16-week intervention study (age 64±6 years, BMI 29.9±4.3 kg/m^2), using a device with vibration feedback to promote physical activity. Before and after intervention, we objectively examined physical activity (ActivPAL), cytokine production capacity after *ex vivo* stimulation in peripheral blood mononuclear cells (PBMCs), metabolism of PBMCs, circulating cytokine concentrations, and monocyte immunophenotype.

Overall, no significant increase in walking time was found (1.9±0.7 to 2.2±1.2 hours/day, *P*=0.07). However, strong, inverse correlations were observed between the change in walking time and the change in production of IL-1β, IL-6, IL-8 and IL-10 after LPS stimulation (r_s=-0.655, -0.844, -0.672, and -0.781, respectively, all *P*<0.05). After intervention optimisation based on feedback from wave 1, participants in wave 2 (n=8) showed an increase in walking time (2.2±0.8 to 3.0±1.3 hours/day, *P*=0.001) and attenuated cytokine production of IL-6, IL-8 and IL-10 (all P<0.05). Glycolysis (*P*=0.08) and maximal OXPHOS (*P*=0.04) of PBMCs decreased after intervention. Lower IL-6 concentrations (*P*=0.06) and monocyte percentages (*P*<0.05), but no changes in monocyte subsets were found.

Conclusion. Successfully improving walking time shifts innate immune function towards a less pro-inflammatory state, characterized by a lower capacity to produce inflammatory cytokines, in individuals with increased CV risk.

Key words: Atherosclerosis, innate immunity, physical activity, sedentary behaviour, cardiovascular disease

This study is registered at the Netherlands Trial Register (NTR6387): https://www.trialregister.nl/trial/6215.

Clinical Perspective

1) What is New?

    - Sedentary behavior (SB) is associated with atherosclerosis and cardiovascular diseases. Low-grade inflammation, largely mediated by monocyte-derived macrophages, contributes to atherosclerosis. Individuals with increased cardiovascular risk due to hypertension or overweight underwent a successful 16 weeks intervention to reduce SB and improve walking time. We showed that effectively reducing SB and improving walking time attenuated cytokine production capacity of circulating mononuclear cells. There was a strong inverse correlations were observed between the change in walking time and the change in production of IL-1β, IL-6, IL-8 and IL-10 of isolated mononuclear cells after ex vivo LPS stimulation.

2) What are the clinical implications?

    - Our results identify a novel mechanism by which less sitting and increased physical activity contributes to improved cardiovascular outcomes.

    - By attenuating cytokine production capacity of circulating innate immune cells, interventions that reduce sitting time could be of benefit in patients in with chronic inflammatory diseases.

Introduction

Atherosclerotic cardiovascular diseases (CVD), including myocardial infarction and stroke, are the major cause of death worldwide [1]. Although regular exercise training is a potent strategy to reduce risk

for future CVD [2], only 50-60% of the Western population meet these guidelines [3] with even lower prevalence rates in those at risk for CVD [4]. In the past decade, an increasing number of studies explored the role of sedentary behaviour (*i.e.* any waking behaviour in a sitting, reclining or lying posture with energy expenditure below 1.5 metabolic equivalents) in the context of CVD. Interestingly, high levels of sedentary behaviour, independent of exercise performance, are associated with an increased risk for all-cause mortality and CVD [5-7]. Cross-sectional studies have linked sedentary behaviour to higher blood pressure [8], BMI [9], waist circumference [9, 10], glucose intolerance [10], and lower high density lipoprotein cholesterol (HDLc) [9]. Despite these cross-sectional observations, little work explored potential underlying mechanisms of the detrimental impact of sedentary behaviour on development of future CVD.

Atherosclerosis is a low-grade inflammatory disorder of the arterial wall, in which monocyte-derived macrophages orchestrate the development of atherosclerotic plaques [11]. We and others have recently described that circulating monocytes of individuals with atherosclerosis have a long-lasting pro-inflammatory phenotype. This is characterised by an enhanced cytokine production capacity and a switch to an increased glycolytic metabolism [12, 13], suggesting that functional and metabolic alterations in circulating monocytes contribute to atherogenesis. Individuals with increased risk for CVD due to dyslipoproteinemia are also characterized by a pro-inflammatory monocyte phenotype [14]. Importantly, a previous study found that a Western type diet in atherosclerosis-prone mice is capable of inducing a persistent pro-inflammatory phenotype of circulating monocytes and their bone marrow progenitors [15]. Furthermore, pharmacological therapy inhibiting interleukin-1β (IL-1β) in the innate immunity pathway successfully reduced the risk of future cardiovascular events in patients with CVD [16]. This suggests that interventions, including pharmacological and environmental strategies that impact on innate immune function, may alter the process of atherosclerosis.

Sedentary behaviour is associated with markers of chronic low-grade inflammation (*e.g.* high concentrations of C-reactive protein (CRP), IL-6, neopterin, leptin, adiponectin and tumor necrosis factor alfa (TNFα)) [17, 18]. However, no previous study directly examined whether a change in sedentary behaviour (or physical activity) results in modulation of the innate immune function. Therefore, we examined the impact of a 16-week intervention to reduce sedentary time and increase physical activity levels on the innate immune function in subjects with increased risk for CVD. For this purpose, we explored the functional and metabolic phenotype of innate immune cells in terms of *ex vivo* cytokine production capacity and intracellular metabolism.

Methods

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

Subjects

Individuals with an increased cardiovascular risk were recruited by newspaper and internet advertisement in Nijmegen, The Netherlands. Individuals aged above 55 years with at least 40 hours per week of self-reported sedentary behaviour were eligible for participation. Criteria for inclusion were the presence of one or more cardiovascular risk factors, consisting of high blood pressure (SBP >160 mmHg, DBP >90 mmHg), anti-hypertensive medication use, or BMI >28 kg/m^2. Exclusion criteria were presence of diabetes mellitus, autoimmune diseases or autoinflammatory diseases, and daily immunomodulatory drug use. Moreover, individuals were excluded if they were not able to perform light-intensity physical activity (e.g. standing and walking) or when unable or not allowed to provide informed consent. The study protocol was approved by the Institutional Review Board Arnhem/Nijmegen, the Netherlands and registered at the Netherlands Trial Register (NTR6387). All individuals gave written informed consent.

Study design

Subjects reported to our laboratory on four measurement days; two test days before and two test days after the 16-week intervention (Figure 1). At each measurement day, participants were instructed to refrain from caffeine and alcohol intake at least 12 hours prior to the test. Moreover, participants were instructed to refrain from vigorous exercise 24 hours prior to the measurements. At the first visit, participant characteristics were assessed and blood was drawn for immunological assessment. In the subsequent week, baseline sedentary behaviour characteristics of the subjects were measured using an activity monitor combined with an inclinometer (ActivPAL, PAL technologies Ltd, Glasgow, UK). At

a second visit, participants handed in the activity monitor, received written and oral information about the reduced sitting intervention, and participants started the 16-week intervention. Physical activity patterns were assessed during the last week of the intervention and thereafter all other measurements were repeated.

Intervention

The 16-week mHealth reduced sitting intervention involved prevention of prolonged sitting behaviour (>30 minutes) throughout the day and promotion of low-intensity physical activity (e.g. walking, standing). Subjects received a pocket pedometer Activ8sit (2M Engineering, Valkenswaard, the Netherlands) which recorded physical activity patterns using an inclinometer and tri-axial accelerometer. This combination of information allows for recognizing prolonged periods of sedentary behaviour and physical activity. A vibrating signal was provided after 30 minutes of SB and served as a reminder to break up sitting by low- to moderate-intensity physical activity for at least 2 minutes. Next to direct feedback, participants were able to review their physical activity patterns in a web-based environment. Furthermore, subjects had a phone meeting with their personal coach periodically to evaluate their participation in the reduced sitting intervention. The intervention study was performed in two groups. The intervention for wave 1 was performed in September 2017 to January 2018 (*i.e.* group1.0, n=8) and wave 2 from March to July 2018 (*i.e.* group2.0, n=8). Based on feedback from the participants in group1.0, coaching and support was intensified for subjects in group2.0 to optimise the intervention and to further reduce sedentary behaviour.

Measurements

Cardiovascular risk assessment

Medical history, smoking status, medication use and BMI were assessed in all participants. Capillary blood was used to measure fasting glucose concentrations. Blood pressure was measured twice by a manual sphygmomanometer in sitting position after 5 minutes rest according to AHA guidelines [19].

Sedentary behaviour

A validated activity monitor (ActivPAL3 micro, PAL technologies, Glasgow, United Kingdom) was used to measure number of steps, sedentary time, standing and walking time [20]. The ActivPAL was attached to the ventral side of the right thigh. The monitor was coated in a waterproof sleeve to enable assessment for 8 days continuously. The first day was excluded for data analyses, leaving 7 days of sedentary behaviour measurements. ActivPAL data was processed using an analysis script in Matlab R2014b (The Mathworks, Inc., Natick, Massachusetts). This allowed distinguishing between wake times and bed times per day for each subject and calculated sedentary time, sedentary breaks, number of prolonged sedentary bouts (>30 minutes), standing, walking time and number of steps per day [21].

Blood sampling

Directly before and after intervention, non-fasting blood was collected in EDTA vacutainers. Sample collection was performed at 8.00-9.00 in the morning to avoid interference of circadian rhythms of immune parameters, and sample processing occurred within 2 hours. Plasma and serum were stored at -80°C until further use. Total cholesterol (Tchol), high-density lipoprotein cholesterol (HDLc), and triglycerides were measured in fasting Lithium Heparin plasma using standardized methods, and low-density lipoprotein cholesterol (LDLc) was calculated with the Friedewald formula. Total blood cell counts were determined with an automated Sysmex-XN 450 hematology analyser (Sysmex, Hamburg, Germany).

PBMC isolation and stimulation

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare, Chicago, IL, USA). Cell composition was evaluated by Sysmex analyser

(Sysmex). PBMCs were concentrated in Roswell Park Memorial Institute 1640 Dutch-modified culture medium (RPMI) (Life Technologies/Invitrogen, Waltham, MA, USA) supplemented with 2 mmol/L glutamine (Invitrogen), 10 mg/mL gentamicin (Centrafarm, Etten-Leur, The Netherlands) and 1 mmol/L pyruvate (Invitrogen). To evaluate cytokine production capacity, 5x10^5 PBMCs per well were stimulated in triplicate for 24 hours in round-bottom 96-well plates (Corning, New York, NY, USA) with the following stimuli: RPMI, 10 ng/mL lipopolysaccharide (LPS) from Escherichia coli serotype 055:B5 [22] (Sigma-Aldrich, St. Louis, MO, USA), 10 µg/mL Pam3CysK4 (P3C) (L2000, EMC microcollections, Tübingen, Germany), 10 µg/mL Resiquimod (R848) (Invivogen, San Diego, CA, USA; Catalog#tlrl-r848-5), 50 µMol C16.0 conjugated with Albumin (Sigma-Aldrich resp. Sanquin, Amsterdam, the Netherlands), and 50 µMol C16.0-Albumin in combination with 300 µg/mL sonicated monosodium urate (MSU) crystals (in house). The preparation of C16.0-Albumin and MSU crystals is previously described [23]. Simultaneously, to assess the adaptive immune response, PBMCs were stimulated in triplicate for 7 days in RPMI, 1×10^6/mL *Candida albicans conidia* (UC820 strain), and 1x10^6/mL *Staphylococcus aureus* (ATCC 29213 strain) both with 10% human pool serum. After the incubation periods of 24 hours and 7 days, supernatants were stored after plate centrifugation at −80°C until cytokine assessment.

Cytokine measurements

In stored supernatants cytokine concentrations were measured using ELISA (Supplemental Table I). Circulating cytokine concentrations were determined using ELLA cartridges for IL-1RA, IL-1β, IL-6 and IL-18 on the ELLA platform (Simpleplex, San Jose, CA, USA). Thiobarbituric Acid Reactive Substances (TBARs) were measured in plasma as measure for oxidative stress (OXitek TBARs Assay kit, ZeptoMatrix, New York, NY, USA).

Flow cytometry

Monocyte subpopulations, platelet complexes and expression markers were identified with flow cytometry. Using the lysis-no-wash strategy (BD Pharm Lyse lysing buffer, Becton Dickinson, Franklin

Lakes, NJ, USA), 50 µL EDTA blood was stained by monoclonal antibodies (CD45 Chrome Orange clone J33 Beckman Coulter, HLA-DR PE clone immu-357 Beckman Coulter, CD14 PC7 clone 61D3 Bioscience, CD16 FITC clone CB16 eBioscience, CD3 APC-750 clone UCTH1 Beckman Coulter, CD56 APC clone N901 Beckman Coulter, CD192 BV421 clone 48607 Becton&Dickinson, CD11b BV785 clone ICRF44 Biolegend, CD41 PC5.5 clone Hip8 Biolegend) and measured with CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). The gating strategy applied is shown in Supplemental Figure I, gates were set with the fluorescence minus one method [24, 25]. Data was analysed with Kaluza 3.1 software (Beckman Coulter). Characterization of monocytes subsets is according to current recommendations [24, 25].

Metabolic measurements

Lactate levels were measured in unstimulated PBMCs after 24 hours. In a subgroup of subjects oxygen consumption rate was measured in freshly isolated 5×10^6 PBMCs collected in RPMI. Oxygen consumption was measured at 37°C using polarographic oxygen sensors in a two-chamber Oxygraph (OROBOROS Instruments, Innsbruck, Austria). First, basal oxygen consumption was measured over a period of ≥10 minutes. Then, leak respiration was measured by adding oligomycin A (2.5 µM), a specific inhibitor of mitochondrial complex V. Next, the mitochondrial uncoupler p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) was added at increasing concentrations (ranging from 0.2 µM to 1.0 µM final concentration) to determine maximal electron transport chain capacity. Finally, non-mitochondrial oxygen consumption was established by adding the mitochondrial complex I inhibitor rotenone (0.5 µM) and the mitochondrial complex III inhibitor antimycin A (2.5 µM).

Statistical analysis

Data are presented as mean ±SD for continuous variables, as number (percentage) for categorical variables and as median [interquartile range] for skewed distributed data. All data were analysed using Wilcoxon signed-rank tests, unless stated otherwise. A 2-sided *P*-value <0.05 was considered

statistically significant. Data were analysed using Prism version 6.0 (GraphPad software, La Jolla, CA, USA) and SPSS version 21.0 (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics

Sixteen participants (age 64±6 years, BMI 29.9±4.3 kg/m^2) participated in the study. After 16 weeks, the increase in walking time (1.9±0.7 to 2.2±1.2 hours/day, *P*=0.07) and step count (8864±4138 to 10656±6821 #/day, *P*=0.06) did not reach statistical significance. The intervention was performed in two waves. Based on feedback from participants in group1.0, coaching and support was intensified for subjects in group2.0 to optimise the intervention. No differences were found between both groups at baseline (Table 1). Importantly, group2.0 (n=8) significantly increased walking time (2.2±0.8 to 3.0±1.3 hours/day, *P*<0.01) and step count (10659±4930 to 14909±7321 #/day, *P*<0.01). No changes were seen in estimated physical fitness. Total cholesterol levels (*P*=0.05) and systolic blood pressure (SBP) (*P*<0.01) decreased in group2.0, whilst group1.0 (n=8) showed a decrease in glucose (*P*<0.01), an increase in BMI (*P*=0.01) and in diastolic BP (*P*=0.01) (Table 1). In addition to analysis of the entire group, sub-analyses on group2.0 (n=8) were performed to specifically examine the impact of reducing sedentary behaviour on innate immune function. All data for group1.0 are reported in Supplemental Tables II-IV.

Circulating inflammatory markers

A trend for a decrease in circulating IL-6 concentration was observed in group 2.0 (n=8) (3.21 to 2.62 pg/mL, *P*=0.08) (Figure 2). The concentrations of inflammatory markers CRP, IL-1β, IL-1RA and IL-18 did not change significantly after intervention. In addition, levels of adiponectin and leptin, both adipokines; TBAR, a marker of oxidative stress; E-selectin and VCAM-1, markers of endothelial dysfunction; and MMP-2, a matrix metalloproteinase, were not affected by the intervention (Supplementary Table V).

Cytokine production capacity of PBMCs

After the intervention a significant reduction in the innate cytokine production capacity of PBMCs was observed in group2.0 (n=8) (Figure 3A). We found an attenuated production of IL-6, IL-8 and IL-10 in PBMCs stimulated for 24 hours with LPS, P3C and R848 (TLR4, TLR2 and TLR7/8 ligands respectively) after the 16-week intervention (all $P<0.05$). For C16.0 stimulation (*i.e.* a saturated fatty acid) an attenuated production in IL-1β, IL-6 and IL-8 (all $P<0.05$) was seen after intervention. After C16.0-MSU stimulation, which induces inflammasome activation, IL-6 and IL-8 production was significantly attenuated after intervention (all $P<0.05$). Correction for monocyte count in the PBMC fraction did not alter the results for cytokine production after LPS and R848 stimulation, and IL-1β production after C16.0 stimulation (see Supplementary Table VI for cell percentages in the PBMC fraction). To study the adaptive immune function, 7 day stimulation of PBMCs with *C. albicans* and *S. aureus* was performed. After intervention, a significant increase in the cytokine response of IFNγ was observed after *C. albicans* stimulation ($P=0.05$), and of IFNγ, IL-22 and IL-17 after *S. aureus* stimulation ($P<0.05$, $P=0.08$, and $P<0.05$, respectively) (Figure 3B).

We examined the correlation between the change in walking time and the change in cytokine production capacity during the intervention on the data of both groups (n=16) as all individuals possess data on a change in walking time and consequent changes in cytokine production capacity (Figure 4). This analysis shows a strong, inverse correlation between IL-1β, IL-6, IL-8 and IL-10 production after stimulation with LPS and R848 *versus* walking time (all $r_s>-0.51$ and $P<0.001$, except IL-8 after LPS $P=0.01$).

Immunophenotyping of monocytes

A reduction in the percentage of circulating monocytes ($P<0.05$) was observed together with an increase in the lymphocyte-to-monocyte ratio ($P=0.01$) after intervention in group2.0 (n=8) (Table 2). The distribution of the monocyte subpopulations, *i.e.* the classical, intermediate and nonclassical monocytes, did not change during the intervention. Also, CCR2 and CD11b expression in monocytes, and monocyte-platelet complexes were not altered (Table 2).

Metabolism of PBMCs

A trend for a decrease in lactate production was observed (P=0.08) after 24 hours in unstimulated PBMCs (Figure 5A). Oxygen consumption in freshly isolated PBMCs tended to decrease after intervention for both basal (P=0.06; n=6) and maximal (P<0.05; n=4) respiration (Figure 5B-C). However, no changes were observed for relative and absolute reserve capacity (P>0.20) (Figure 5D-E).

Discussion

Our main finding is that a 16-week intervention to reduce sedentary behaviour and improve walking time alters the innate immune function towards a less pro-inflammatory state in individuals with increased CV risk. In support of this observation, we found a strong, inverse correlation between the increase in walking time and attenuated cytokine production capacity that followed a dose-response like pattern. In other words, individuals who had the highest increase in walking time, demonstrated the strongest reduction in cytokine production capacity of circulating PBMCs. The reduction in inflammatory state after the 16-week intervention coincided with a trend to decreased glycolysis and oxidative phosphorylation rate. Together, our study provides a mechanistic explanation of the cardiovascular benefits of long-term reductions in sedentary behaviour.

Given the current lack of validated strategies to reduce sedentary behaviour, we have co-developed a physical activity-monitor with the capacity to provide tactile feedback for the purpose of a "reduce sedentary behaviour"-intervention. During this process, data from group1.0 showed no significant improvement in physical activity patterns. Based on feedback from these participants, we adjusted the intervention, largely through an increased frequency and more detailed feedback to the participants (including weekly online or oral feedback). As a result, successful improvement of physical activity after the 16-week intervention in participants from group2.0 was achieved, as supported by the increase in walking time of 45 minutes/day. The difficulty of changing daily sedentary behaviour is illustrated by previous 3-4 month intervention studies, also using mHealth-devices, that failed to markedly improve walking time [26] or sedentary time [27]. This emphasises the importance for (co-)developing interventions, using participants' feedback, to successfully alter sedentary behaviour patterns. Another important observation in our study was that the reduction in sedentary behaviour was achieved through elevation in walking time rather than via increased engagement in exercise training and/or physical fitness. Previous work linked regular exercise training and/or higher fitness to mechanisms of cardiovascular protection and prevention of atherosclerosis [28]. Therefore, the absence of changes in

physical fitness and/or exercise training, allows us to relate the improved innate immune response to the reduction in sedentary behaviour.

The key finding of our study is that reducing sedentary behaviour across a 16-week intervention is associated with a significant reduction in cytokine production capacity of PBMCs (*i.e.* attenuated innate immune response). In previous cross-sectional studies, sedentary behaviour or physical inactivity was found to be associated with several circulating markers of systemic inflammation (CRP, IL-6, TNF-α, neopterin) [17, 18], but the effect on innate immune cell function was unexplored. Our observations confirm these previous findings in that we found a trend for reduction in circulating IL-6 concentrations within individuals after the 16-week intervention.

Next, one could hypothesize about the potential underlying mechanisms driving the attenuated innate immune state. Previous work on the acute impact of sedentary behaviour found marked reduction in vascular function, which was linked to an decreased in arterial blood flow and presence of oxidative stress [29]. More specifically, vitamin C supplementation prevented the impact of 3h sitting on vascular function. However, the investigators found no changes in the circulating oxidative stress markers [30]. In our study we examined a circulating marker of oxidative stress, but neither did we observe significant changes after intervention. Additional markers of antioxidants and oxidative damage are needed to fully exclude a role of oxidative stress. In addition, it is important to note that we cannot exclude the potential presence of local changes of oxidative stress in tissues (e.g. active areas).

Another explanation for our observations, might relate to the metabolic effects of sedentary behaviour versus low-intensity physical activity. A previous study found that an acute sedentary bout impaired glucose metabolism [31]. Interestingly, we found a trend for decreased glycolysis and oxidative phosphorylation rates in PBMCs after our intervention. Recent studies have shown that the hyper-responsiveness of circulating monocytes in terms of cytokine production in patients with atherosclerosis coincides with an increased glycolytic metabolism [12, 13]. Therefore, metabolic changes

during our intervention might contribute to the attenuated inflammatory innate immune function. Moreover, the leptin/adiponectin pathway seems related to the level of physical (in)activity in cross-sectional observations [17, 32, 33]. In contrast to these studies, we found no change in the ratio after the intervention. Together, the decrease in cellular metabolism is one mechanism driving the attenuated immune response after intervention. Still future studies are warranted to better understand our novel observation of the capacity of low-intensity physical activity to attenuate innate immune responses of PBMCs in humans.

Interestingly, in conjunction with an attenuated innate immune response of PBMCs after the intervention, we observed an enhanced production by the adaptive immune cells, as IFNɣ and IL-17 production after S. aureus stimulation significantly increased after the 16-week intervention. A similar counter-regulatory mechanism has been reported previously. A decreased production capacity of IFNɣ was found in patients with cerebral small vessel disease in conjunction with an increased innate immune cytokine production [34]. This increase in the adaptive immune response may represent a counter-regulatory mechanism to compensate for the attenuate innate immune response.

Our results are in line with recent studies that have demonstrated that environmental factors can persistently modulate the inflammatory function of circulating monocytes and, consequently, contribute to changes in risk for future CVD [35]. For example, sleep fragmentation leads to an increase in pro-inflammatory monocytes and subsequently to larger atherosclerotic lesions in mice [36]. In addition, chronic stress induces monocytosis via accelerated haematopoiesis that subsequently promotes vulnerable plaque lesions [37]. Furthermore, a Western-type diet modulates towards long-lasting innate immune reprogramming in myeloid cells and progenitor cells in *Ldlr-/-* mice [15]. In agreement with these earlier observations, our results imply that changes in lifestyle may reduce the risk to develop CV disease through their effects on innate immune function in humans.

Our study may have implications for public health. The ability to successfully reduce sedentary behaviour, in a population at risk for CVD and *a priori* high levels of sedentary behaviour, and the associated diminished pro-inflammatory phenotype of innate immune cells is of potential clinical relevance. More importantly, our work shows that the detrimental effects of sedentary behaviour on atherosclerosis and CVD may, at least partly, be explained through the direct effects on the innate immune function. However, some limitations must be considered.

A potential limitation of this study is that, due to the design of our study, we cannot exclude the impact of external modulators of immune function, *e.g.* seasonality. Previously, annual seasonal variability in cytokine production has been described to significantly peak in summer for several cytokines (*e.g.* TNF-α, IL-1β, and IL-6) after stimulation [38]. We observed a decreased cytokine production after the physical activity intervention in July, which is exactly opposite effect of the annual seasonal summer peak. Moreover, no seasonal effect was found for the cytokine production after P3C and LPS stimulation, the stimuli applied in our study. This suggests that the effect of the intervention unlikely can be explained by seasonality per se. Also, the strong correlation between walking time and cytokine production capacity strongly argues for a direct effect of the intervention on cytokine production capacity. Fluctuations of immune cells over time might have influenced our results, however correction for the monocyte percentage of the PBMC fraction did unalter our strongest findings, namely the cytokine production capacity after LPS and R848 stimulation.

Our relatively small sample size should be considered, although power analysis revealed intermediate (β>0.65) to good (β>0.80) power for most outcomes, except for IL-8 which is underpowered (see Supplemental table VII) and therefore has a larger chance of a type II error (*i.e.* false negative results). Still we were able to detect a significant difference in IL-8 before and after intervention. Nevertheless, caution should be taken extrapolating our results to other groups and/or physical activity interventions.

This study is the first to reveal a diminished pro-inflammatory phenotype of innate immune cells after a successful 16 weeks reduced sitting intervention in subjects with increased cardiovascular risk. These findings, combined with the within-subject approach of our study, support the presence of a dose-response relationship between sedentary behaviour and innate immune response. Given the central role for activated monocytes in atherosclerosis and development of cardiovascular diseases, our observations may have important clinical implications. These data improve our understanding of the link between sedentary behaviour and cardiovascular disease and support the concept that targeting sedentary behaviour may be an important approach in preventing atherosclerotic cardiovascular disease.

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Figures legends

Figure 1. Study design. Measurements were performed across 2 waves (n=8 per wave). Participants visited our laboratory four times; two test days before and two test days after the 16-week intervention.

Figure 2. Circulating cytokines. Individual changes in circulating cytokines before (pre) and after (post) intervention (n=8). Mean is represented in black. Lowest detection limit for IL-1β is 0.31 pg/mL, and for IL-6 1.35 pg/mL, indicated by the dotted line.

Figure 3A-B. Cytokine production capacity of PBMCs. A. Innate cytokine production after 24 hour stimulation (n=8). B. Adaptive cytokine production after 7 day stimulation (n=8). Data before (white) and after intervention (grey) are shown in boxplots (median ± interquartile range). Whiskers represent 95% confidence interval. * indicates $P<0.05$.

Figure 4. Cytokine production capacity correlated with walking time per day (n=16). The change in cytokine concentration after LPS (black) and R848 (red) stimulation correlated with the change in walking time. Linear regression with 90% confidence interval. * indicates $P<0.05$, ** $P<0.01$, r_s: Spearman correlation coefficient.

Figure 5A-E. Metabolism of PBMCs: A. Extracellular lactate production in unstimulated PBMCs (n=8). B. Representative result of the OROBOROS. C. Oxygen consumption is presented as basal respiration (basal) (n=6), proton leak after inhibition of ATP synthase (leak) (n=4), maximal oxygen consumption (max) (n=4), residual oxygen consumption or non-mitochondrial oxygen consumption (rox) (n=6). D-E. Absolute and relative oxygen consumption reserve (n=4). Data before (white) and after intervention (grey) are shown in boxplots (median ± interquartile range). Whiskers represent 90% confidence interval. * indicates $P<0.05$.

Tables

Table 1. Participants' characteristics.

Baseline characteristics	**Total (n=16)**		**Group1.0 (n=8)**		**Group2.0 (n=8)**	
Sex (% male, n)	31 (5)		25 (2)		38 (3)	
Age (years)	64±6		66±5		62±6	
Current smoking (%, n)	13 (2)		0 (0)		25 (2)	
Hypertension (%, n)	69 (11)		50 (4)		88 (7)	
Lipid lowering therapy (%, n)	25 (4)		25 (2)		25 (2)	
B-antagonist use (%, n)	31 (5)		38 (3)		25 (2)	
Intervention outcomes	Pre	Post	Pre	Post	Pre	Post
SBP (mmHg)	132±10	131±9	128±11	134±8	**136±7**	**128±10****
DBP (mmHg)	**82±7**	**84±7***	**80±7**	**84±6***	83±7	83±8
BMI (kg/m2)	29.9±4.3	30.0±4.2	**30.2±2.6**	**30.6±2.6***	29.5±5.7	29.4±5.4
Glucose (mmol/L)	**6.17±0.77**	**5.78±0.74***	**6.43±0.67**	**5.96±0.72****	5.91±0.82	5.61±0.76
Tchol (mmol/L)	5.11±0.74	4.88±0.61	5.04±0.76	4.94±0.48	**5.18±0.78**	**4.83±0.75***
HDLc (mmol/L)	1.43±0.35	1.39±0.32	1.37±0.40	1.33±0.33	1.49±0.31	1.45±0.33
LDLc (mmol/L)	2.85±0.72	2.77±0.60	2.74±0.74	2.80±0.64	2.96±0.72	2.74±0.60
Triglycerides (mmol/L)	1.84±1.11	1.63±0.93	2.06±1.14	1.82±1.00	1.62±1.12	1.43±0.87
Non-HDLc (mmol/L)	3.67±0.69	3.51±0.52	3.66±0.82	3.63±0.43	3.68±0.60	3.40±0.61
Sedentary time (hours/day)	10.1±1.3	9.8±1.5	10.4±1.7	10.3±1.5	9.9±0.6	9.3±1.4
Sitting time >30 min (#/day)	6±2	6±2	7±2	7±1	5±1	5±1
Walking time (hours/day)	1.9±0.7	2.2±1.2	1.5±0.5	1.4±0.4	**2.2±0.8**	**3.0±1.3****
Total step count (#/day)	8864±4138	10656±6821	7069±2243	6402±2181	**10659±4930**	**14909±7321****
Estimated physical fitness (ml O_2/ml/kg)	27.7±7.4	27.7±8.8	25.3±5.4	22.6±4.9	30.0±8.6	32.8±9.1
Central vascular stiffness†	7.8±1.7	7.6±2.9	n.a.	n.a.	7.8±1.7	8.1 ±2.8
Peripheral vascular stiffness†	9.1±1.0	9.0±2.4	n.a.	n.a.	9.1±1.0	9.8 ±1.3

† data is missing for 8 participants. * indicates $P< 0.05$, **: $P<0.01$. Paired samples T-test, mean, SD, or X^2 test for categorical data, mean (n). First and second group were matched (age, sex, BMI, smoking). SBP indicates systolic blood pressure, DBP: diastolic blood pressure.

Table 2. Circulating cell counts and immunophenotyping of monocytes (n=8).

Cell counts	**Pre**	**Post**
WBC (10^6/mL)	5.3 [4.4-6.1]	5.0 [4.5-6.2]
Neutrophils (10^6/mL)	2.9 [2.6-3.3]	2.8 [2.4-3.2]
Lymphocytes (10^6/mL)	1.7 [1.3-2.2]	1.7 [1.6-2.5]
Monocytes (10^6/mL)	0.50 [0.37-0.60]	0.39 [0.27-0.54]
Monocytes (%)	**9.0 [7.3-10.3]**	**7.9 [6.1-8.7]***
Lymphocyte monocyte ratio	**3.47 [2.95-4.51]**	**4.59 [3.90-6.13]***
Neutrophil lymphocyte ratio	1.54 [1.41-2.46]	1.61 [1.21-1.83]
Immunophenotype monocytes		
Monocyte subsets (% total)	7.5 [6.0-7.9]	5.7 [3.8-6.6]
Classical Mo (% gated)	78.5 [76-81]	79.0 [75-84]
Intermediate Mo (% gated)	7.3 [4.4-8.1]	7.0 [4.1-8.0]
Nonclassical Mo (% gated)	14.1 [13.0-16.1]	12.7 [10.4-18.4]
CD41+ Mo (% gated)	8.0 [7.0-8.7]	8.5 [8.0-9.2]
CCR2+ Mo (MFI)	5255 [4607-5932]	4385 [3894-6873]
CD11b+ Mo (MFI)	8872 [6721-10893]	10743 [7924-10978]

WBC indicates white blood cell counts, Mo: monocytes, MFI: mean fluorescence intensity. * indicates $P < 0.05$. Median, IQR.