**Supplemental methods, SDC 1**

**Participants**

Individuals with an increased cardiovascular risk were recruited by local newspaper and internet advertisement in the direct environment of Nijmegen, the Netherlands. Individuals aged ≥55 years with >40 hours per week of self-reported sedentary behavior were eligible for participation. Criteria for inclusion were the presence of one or more cardiovascular risk factors, consisting of BMI >28 kg/m2, high blood pressure (SBP >160 mmHg, DBP >90 mmHg) and anti-hypertensive medication use. Individuals were excluded if they were not able to perform light-intensity physical activity (*i.e.* standing and walking) or to provide informed consent. The study protocol was approved by the local ethics committee (CMO region Arnhem Nijmegen, the Netherlands) and registered at the Netherlands Trial Register ([NTR6387](https://www.trialregister.nl/trial/6215)). All individuals provided written informed consent. Measurements were performed between 2017 and 2019. A subset of this study answering a different research question was recently published elsewhere 1.

**Study design**

Each subject reported in 3 clusters of 3 measurement days to our laboratory: a first cluster before a 16-week control period (T0), a second cluster after the 16-week control period (T1) and, finally, a third cluster after a 16-week intervention period (T2) (Figure 1). Measurements at T0 were performed as familiarization sessions for the participants and to minimize measurement variation in outcomes. On Day 1 and 2, peripheral vascular and cerebrovascular blood flow and function were assessed at baseline. Subsequently, in randomized cross-over order between Day 1 and 2, subjects underwent a 3-hour sitting trial without moving their lower extremities (SIT), and a 3-hour sitting trial with 2-minute light-intensity walking breaks at self-selected pace every 30 minutes (BREAKS). Immediately following the 3-hour period, peripheral vascular and cerebrovascular flow and function were assessed again. Finally, at Day 3 baseline characteristics and physical fitness were assessed. Physical activity monitors were mounted to assess physical activity and sedentary behavior characteristics across a 8-day period. The same set of measurements was repeated at T1 and T2.

**Intervention.** The 16-week reduced sitting intervention aimed to prevent prolonged sitting time (>30 minutes) throughout the day and to promote low-intensity physical activity (*e.g.* walking, standing). Subjects received information regarding the purpose of the intervention and wore a customized activity monitor to objectively monitor sedentary behavior (Activ8sit, 2M Engineering, Valkenswaard, the Netherlands, Supplemental Figure 1). This pocket-worn device consists of an inclinometer and a tri-axial accelerometer, which allows for recognizing prolonged periods of sedentary behavior and physical activity patterns. Upon recording prolonged, uninterrupted sitting (*i.e.* 30-minutes), vibrotactile feedback was provided by the monitor to remind participants to replace sedentary behavior by low-intensity physical activity (*e.g.* walking, standing). Participants were able to review their physical activity patterns in a web-based environment. Regular phone meetings with a researcher were made to evaluate participation. To bypass previously identified problems when translating interventions to reduce sedentary behavior 2, we adopted an embedded pilot study-design, where input of participants was used to optimize the 16-week intervention for the subsequent groups of participants (*i.e.* waves). Following this approach, the intervention was performed in three waves. Participants were assigned to wave 1.0 based on order of application. Based on feedback from the participants in wave 1.0, coaching and support was intensified to weekly meetings (phone or online) for subjects in wave 2.0 and 3.0 and a half-way group-meeting was organized to optimize the intervention and to further reduce sedentary behavior. Subsequently, participants were randomly assigned to wave 2.0 or 3.0. Intervention was performed in September 2017 to January 2018 (for wave 1.0, n=9), March to July 2018 (for wave 2.0, n=9), and October 2018 to February 2019 (for wave 3.0, n=8).

**Measurements**

Before 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. All vascular measurements were performed according to guidelines to assess peripheral vascular function 3.

**Participant characteristics.** Medical history, medication use and BMI were assessed in all participants. Capillary blood was used to measure fasting glucose levels. Fasting venous blood was collected in Lithium Heparin vacutainers and sample processing occurred within 30 minutes. Plasma was stored at -80°C until further use. Insulin, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured in fasting lithium heparin plasma using standardized methods, and low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula. As an explorative outcome, HOMA-IR was calculated from glucose and insulin levels in wave 2.0 and 3.0. Blood pressure was measured twice by a manual sphygmomanometer after 5 minutes seated rest according to AHA guidelines 4. The Astrand-Rhyming test was used as a submaximal cycling test to estimate physical fitness, expressed as estimated maximal oxygen consumption 5.

**Physical activity patterns.** A validated activity monitor (ActivPAL3 micro, PAL technologies, Glasgow, United Kingdom) was used to measure physical activity patterns 6. As the ActivPAL combines a tri-axial accelerometer with an inclinometer, the ActivPAL is able to distinguish between sitting, standing and walking. The first day was excluded for data-analyses. ActivPAL data was processed using a validated analysis script in Matlab R2014b (The Mathworks Inc., Natick, MA, USA) 7. Sedentary, standing, and walking time, sedentary breaks, number of sedentary bouts (>30 min), and steps per day were computed.

**Peripheral vascular blood flow and function.**Superficial femoral artery (SFA) flow-mediated dilation (FMD) was measured as a test of peripheral vascular function 8. After a resting period of 10 minutes in the supine position, the right SFA was examined ~3 cm distal from the bifurcation using a 10‐MHz multifrequency linear array probe attached to a high‐resolution ultrasound machine (T3000, T3300; Terason, Burlington, MA, USA). Continuous Doppler velocity assessment was simultaneously obtained using the lowest possible insonation angle (always <60°). After a 1-minute resting period, a blood pressure cuff was inflated to supra-systolic pressure for 5 minutes. Recording of the diameter and blood velocity resumed 30 seconds prior to deflation and continued for 5 minutes. Analysis of SFA diameter, blood flow and shear rate was performed using custom‐designed edge‐detection and wall‐tracking software 9,10. Peak diameter after cuff deflation was automatically detected according to an algorithm as described elsewhere 11.

**Cerebrovascular blood flow and function.** Continuous blood pressure was measured in the middle finger of the right hand using photoplethysmography (Finapres Medical Systems, Amsterdam, the Netherlands). An arm sling was used to stabilize the hand at heart level. Heart rate was monitored using a three-lead electrocardiogram (BIOPAC Systems, Goleta, CA, USA). Cerebral blood flow velocity (CBFv) in the middle cerebral arteries was measured using transcranial Doppler ultrasonography. Once the left and right middle cerebral arteries were identified through the temporal windows by two 2-MHz probes (Multi-Dop, Compumedics DWL, Singen, Germany), the signal was optimized by adjusting gain, depth, angle and position, and the probes were fixed with a Spencer head frame (Spencer technologies, Seattle, WA, USA). A nasal cannula was used to monitor exhaled CO2 by capnography (BIOPAC Systems). All signals were recorded at 200 Hz using a data acquisition system (MP150, BIOPAC Systems).

During 5 minutes sitting, resting CBFv was measured. Hypocapnia was induced by hyperventilating at a frequency of 0.5 Hz (1 second breathing in, 1 second breathing out) for 30 seconds. After 2 minutes rest, hypercapnia was induced by inhalation of a gas mixture with steadily increasing concentrations of CO2 (30 seconds 3%, 30 seconds 4%, 4 minutes 5%). Using these values, cerebral vasomotor reactivity (CVMR), the change in CBFv to changes in arterial CO2 concentration, could be determined 12. CVMR was computed by the difference between maximal cerebrovascular conductance index (CVCi, *i.e.* the ratio of CBFv and mean arterial pressure) during hypercapnia and minimal CVCi during hypocapnia, divided by the mean CVCi during normocapnia. Cerebrovascular conductance index was used to account for confounding effects of CO2 on blood pressure 13.

Repeated sit-to-stand maneuvers (10 seconds sitting, 10 seconds standing) for 5 minutes were used to enhance very low frequency hemodynamic fluctuations at 0.05 Hz 14. Using these fluctuations, cerebral autoregulation (CA) could be computed via transfer function analysis, resulting in gain, normalized gain (nGain) phase and coherence as output. Gain is the damping of fluctuations in blood pressure in CBFv (lower gain indicates better CA). Phase represents the time shift in CBFv adaptations to blood pressure fluctuations (higher phase indicates active adaptation as seen in normal CA). Coherence is a measure for coherence between CBFv and blood pressure and serves as a measure of data quality 15. These parameters were averaged over the very low-frequency (0.02–0.07 Hz), where CA is most active 15. Next to CA, cardiac baroreflex sensitivity (BRS) was calculated by transfer function analysis using SBP and R-R interval as input 16. Here, a higher gain indicates better BRS. Because the 0.05 Hz repeated sit-stand manoeuvers could influence the hemodynamic effects of prolonged sitting, as they require repeated active standing, we also analyzed CA and BRS using transfer function analysis in slow sit-stand maneuvers. These measurements were performed preceding the 0.05 Hz repeated sit-stand manoeuvers, and consisted of a data segment of 9 minutes (3 periods of 2 minutes sitting and 1 minute standing) 16.

Beat-to-beat data of all cerebrovascular measurements were pre-processed and analyzed using custom-written Matlab scripts (version 2014b, the MathWorks Inc.) as previously described by de Jong *et al.* 16.

**References**

1. Noz MP, Hartman YAW, Hopman MTE, et al. Sixteen-Week Physical Activity Intervention in Subjects With Increased Cardiometabolic Risk Shifts Innate Immune Function Towards a Less Proinflammatory State. *J Am Heart Assoc.* 2019;8(21):e013764.

2. Stephenson A, McDonough SM, Murphy MH, Nugent CD, Mair JL. Using computer, mobile and wearable technology enhanced interventions to reduce sedentary behaviour: a systematic review and meta-analysis. *Int J Behav Nutr Phys Act.* 2017;14(1):105.

3. Thijssen DHJ, Bruno RM, van Mil A, et al. Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. *Eur Heart J.* 2019.

4. Williams B, Mancia G, Spiering W, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. *Eur Heart J.* 2018;39(33):3021-3104.

5. Astrand PO, Ryhming I. A nomogram for calculation of aerobic capacity (physical fitness) from pulse rate during sub-maximal work. *J Appl Physiol.* 1954;7(2):218-221.

6. Lyden K, Kozey Keadle SL, Staudenmayer JW, Freedson PS. Validity of two wearable monitors to estimate breaks from sedentary time. *Med Sci Sports Exerc.* 2012;44(11):2243-2252.

7. van der Berg JD, Willems PJ, van der Velde JH, et al. Identifying waking time in 24-h accelerometry data in adults using an automated algorithm. *J Sports Sci.* 2016;34(19):1867-1873.

8. Kooijman M, Thijssen DH, de Groot PC, et al. Flow-mediated dilatation in the superficial femoral artery is nitric oxide mediated in humans. *J Physiol.* 2008;586(4):1137-1145.

9. Woodman RJ, Playford DA, Watts GF, et al. Improved analysis of brachial artery ultrasound using a novel edge-detection software system. *J Appl Physiol (1985).* 2001;91(2):929-937.

10. Pyke KE, Dwyer EM, Tschakovsky ME. Impact of controlling shear rate on flow-mediated dilation responses in the brachial artery of humans. *J Appl Physiol (1985).* 2004;97(2):499-508.

11. Black MA, Cable NT, Thijssen DH, Green DJ. Impact of age, sex, and exercise on brachial artery flow-mediated dilatation. *Am J Physiol Heart Circ Physiol.* 2009;297(3):H1109-1116.

12. Glodzik L, Randall C, Rusinek H, de Leon MJ. Cerebrovascular reactivity to carbon dioxide in Alzheimer's disease. *J Alzheimers Dis.* 2013;35(3):427-440.

13. Claassen JA, Zhang R, Fu Q, Witkowski S, Levine BD. Transcranial Doppler estimation of cerebral blood flow and cerebrovascular conductance during modified rebreathing. *J Appl Physiol (1985).* 2007;102(3):870-877.

14. Smirl JD, Hoffman K, Tzeng YC, Hansen A, Ainslie PN. Methodological comparison of active- and passive-driven oscillations in blood pressure; implications for the assessment of cerebral pressure-flow relationships. *J Appl Physiol (1985).* 2015;119(5):487-501.

15. Claassen JA, Meel-van den Abeelen AS, Simpson DM, Panerai RB, international Cerebral Autoregulation Research N. Transfer function analysis of dynamic cerebral autoregulation: A white paper from the International Cerebral Autoregulation Research Network. *J Cereb Blood Flow Metab.* 2016;36(4):665-680.

16. de Heus RAA, de Jong DLK, Sanders ML, et al. Dynamic Regulation of Cerebral Blood Flow in Patients With Alzheimer Disease. *Hypertension.* 2018;72(1):139-150.