ONLINE SUPPLEMENT

Impact of obstructive sleep apnea and intermittent hypoxia on blood rheology - a translational study

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Materials and methods

Animal model of intermittent hypoxia (IH)

All animal experiments were performed in Grenoble (France; 212m asl) in accordance with the Institute for Laboratory Animal Research "Guide for the Care and Use of Laboratory Animals" and received approvals (n° 201603301129626) from the Université Grenoble Alpes Ethical Committee and the French ministry. Male Wistar rats (n=15) were after randomization exposed to normoxia (n=7) or IH (n=8) for 14 days, 8 hours per day, while the animals were sleeping. The IH stimulus consisted in 60s cycles alternating 30s of hypoxia (Fraction of inspired O_2 - FiO_2=5%) and 30s of normoxia (FiO_2=21%). Rats exposed to normoxia were exposed to similar cycles, but the F_iO_2 was maintained at 21% for both cycles. This was done to reproduce noises and turbulences related to gas circulation while keeping the rats normoxic. After 14 days of IH, rats were anesthetized using intraperitoneal injection of pentobarbital ($60mg \cdot Kg^{-1}$). Then heparin was intravenously administered to prevent subsequent blood coagulation. Finally, blood was drawn to measure hemorheological parameters and redox balance.

Human experiments

This study component was performed according to the *Declaration of Helsinki* and was approved by the Conjoint Health Research Ethics Board of the University of Calgary (REB15-1153).

The human experiments were performed in Calgary (Alberta, Canada; 1103m asl) using data collected from a sub-sample of participants within a larger study examining the impact of nocturnal oxygen therapy and CPAP on cerebrovascular and cardiorespiratory regulation in OSA [1, 2]. Twenty-three newly diagnosed patients with OSA and 13 healthy control participants, between 18 and 66 years, were recruited from referrals to the Sleep Centre at

Foothills Medical Centre (Calgary, Alberta), respiratory home care providers, and the local community. OSA was diagnosed by unattended home-based nocturnal cardio-pulmonary monitoring (home sleep apnea test (HSAT) [3]; Remmers Sleep Recorder; Sagatech Electronics, Calgary, Alberta). This system calculates an oxygen desaturation index (ODI) by indexing the number of times arterial oxygen saturation (SaO_2) decreases by ≥4% to the total Sa_{O2} recording time. Raw data from the sleep recorder was reviewed by a sleep medicine physician (PJH) to confirm the presence or absence of sleep apnea and nocturnal hypoxia.

As nocturnal oxygen will decrease the ODI calculated in this way by preventing Sa_{O2} from decreasing even though apneas and hypopneas continue to occur, a respiratory event index (REI) was also determined by a registered polysomnography technologist (JKR). This index was obtained by manually counting the number of times that nasal air flow decreased by >30% for 10s or longer divided by total air flow recording time [4] . JKR was blinded to all channels (i.e., heart rate, SaO_2, position, snoring) except for airflow. We recruited moderate-to-severe OSA patients (ODI ≥15events·hour^{-1}) with significant nocturnal hypoxemia (mean SaO_2 <90% for ≥12% of total recording time). Control participants had an ODI ≤10events.hour^{-1} and a mean SaO_2 <90% for <12% of total recording time. To specifically study the effect of OSA on blood rheological parameters we removed confounders by excluding patients and controls with a history of stroke, peripheral vascular disease, coronary artery disease, hypertension (systolic/diastolic blood pressure ≥140/90 mmHg or use of hypertensive medication), renal dysfunction (estimated glomerular filtration rate ≤60mL·min^{-1}·1.73m^{-2}), history of diabetes (or a fasting glucose ≥7.0mmol·L^{-1}), and severe asthma as well as other respiratory disorders (e.g., chronic obstructive pulmonary disease). Patients and controls were also excluded if they had a BMI ≥40kg·m^{-2}, were pregnant, a current smoker, or were taking medications known to affect the vasculature such as statins. Participants who met inclusion/exclusion criteria were

contacted, provided with an overview of the study protocol, potential risks and gave written informed consent prior to visiting the research laboratory.

Human experimental protocol

Human experimental protocol is shown in supplemental Figure E1.

***OSA patients*.** Patients performed a familiarization visit and three experimental visits. Twenty-three patients performed visit 1, which determined baseline blood rheology and redox balance. Following visit 1, patients were randomized into one of two groups - one group used nocturnal O_2 for 2 weeks (Oxygen; n=13); the other group received no treatment for OSA for 2 weeks (Air; n=10). After 2 weeks, the Air and Oxygen groups performed the second experimental visit. Preceding this visit, Oxygen patients performed another HSAT while using oxygen to confirm correction of OSA-related hypoxemia. After visit 2, all OSA patients received CPAP and underwent visit 3 after ~4 weeks of adherent treatment (post-CPAP). Preceding visit 3, patients underwent a final HSAT on their prescribed level of CPAP to confirm correction of OSA (ODI $\leq$5events·hour^{-1}) and nocturnal hypoxemia (mean SaO_2 <90% for <12% of total recording time). Between visit 2 and visit 3 (post-CPAP), 6 patients were lost to follow-up: 5 were non-adherent to CPAP and 1 initiated hypertensive medication. Good adherence was defined as CPAP use for $\geq$4h·night^{-1} for $\geq$70% of the days over 4 consecutive weeks [5] monitored by electronic download.

Control participants. Control participants performed a familiarization visit and two experimental visits. The first (baseline) and second (follow-up) experimental visits were to establish baseline blood rheological parameters and to control for a potential time effect within the OSA patients, respectively. The thirteen controls returned for a follow-up test 70 to 228 days later (mean ± SD: 115 ± 44). Follow-up visits were scheduled to correspond approximately with the interval between visit 1 (baseline) and visit 3 (post-CPAP) for OSA patients (47 to 273 days; mean ± SD: 154 ± 60).

Blood pressure assessment in OSA and control participants

During visit 1, brachial blood pressure (BP755, Omron Healthcare, Burlington, Ontario) was measured in a sitting position at least three times throughout a 10-min resting period. Subsequently, these blood pressures were averaged.

Hemorheological and hematological parameters

Non-fasting venous blood samples were drawn and placed in EDTA tubes for hematological and hemorheological measurements. All hemorheological measurements were performed according to the international guidelines for hemorheological laboratory techniques [6]. Hemorheological parameters were measured within four hours of blood sampling and after full re-oxygenation of blood for 10-15min [6].

Rats. Hematocrit was measured by microcentrifugation. Blood and plasma viscosity were measured at 25°C using a cone-plate viscometer (Anton Paar MCR 302 with CPE 50 spindle). Blood viscosity was determined at low ($2.15s^{-1}$) and high shear rates ($1000\ s^{-1}$) and plasma viscosity at $1000\ s^{-1}$. Blood viscosity was determined on native blood and a second time after normalizing blood at a hematocrit of 40%.

Humans. Hematocrit was measured using a blood gas analyzer (ABL837 FLEX, Radiometer, Denmark). Blood viscosity was measured at ~23°C using a cone-plate viscometer (Brookfield DV2T with CPE40 spindle) at low ($45s^{-1}$) and high shear rates ($225s^{-1}$). The erythrocyte elongation index values were determined at 3 and 30 Pa by laser diffraction analysis (ektacytometry) and at 37°C, using the Laser assisted Optical Rotational Cell Analyzer (LORRCA MaxSis, RR Mechatronics, Hoorn, The Netherlands). The system calculates an average erythrocyte elongation index. The higher this index, the more deformable the erythrocytes. Next, after adjustment of the blood sample hematocrit to a standardized 40%,

erythrocyte aggregation index was determined at 37°C via syllectometry (*i.e.*, laser backscatter versus time), using the LORRCA MaxSis. The higher this index, the faster erythrocytes aggregate together. The disaggregation threshold (*i.e.*, the minimal shear rate needed to prevent erythrocyte aggregation or to break down existing erythrocyte aggregates) was determined using a re-iteration procedure [7]. For erythrocyte deformability measurements, red blood cells were resuspended in polyvinylpyrrolidone. Total counts of white blood cells and platelets, hemoglobin concentration, mean cell volume and mean cell hemoglobin concentration were determined at baseline using a hematology analyzer (Max M-Retic, Coulter, USA).

For both rats and humans blood viscosity was measured at low shear rate and high shear rate. For a given hematocrit, blood viscosity at very low shear rate is strongly impacted by erythrocyte aggregation, whereas high shear rate blood viscosity reflects primarily erythrocyte deformability. The different shear rates used in rats (Grenoble) and humans (Calgary) analyses were due to the different capabilities of viscometers used at each location but do not have an impact on physiological interpretations.

Blood viscosity was measured at 23-25°C in our experiments and not at 37°C. However, temperature has a non-linear and major effect on blood viscosity for temperature ranging from 0 to 15°C, whereas this effect is small and linear between 25 and 37°C [8]. Hence, measuring blood viscosity at 25°C rather 37°C should not have affected the comparison between groups in the present study.

Assessment of plasma redox balance in rats and humans

Plasma was separated from blood by microcentrifugation.

***Antioxidant enzymes*.** The quantitative determination of the superoxide dismutase (SOD) activity was performed using the Beauchamps and Fridovich's method [9], slightly modified

by Oberley and Spitz [10]. SOD activity was determined by the degree of inhibition of the reaction between superoxide radicals, produced by a hypoxanthine-xanthine oxidase system, and nitroblue tetrazolium. Plasmatic Gluthatione peroxidase (GPX) was determined by the modified method of Paglia and Valentine [11], using H_2O_2 as a substrate. GPX was determined by the rate of oxidation of NADPH to NADP after addition of glutathione reductase (GR), reduced glutathione (GSH), and NADPH.

Oxidative stress biomarkers. Plasmatic malondialdehyde (MDA) concentration was determined as thiobarbituric reactive substances by a modified method of Ohkawa et al. [12]. MDA is a marker of lipid peroxidation. Plasmatic advanced oxidation protein products (AOPP) were determined using the semi-automated method described by Witko-Sarsat *et al*. [13]. AOPP were measured by spectrophotometry and were calibrated with chloramine-T solution that absorbs at 590 nm in the presence of potassium iodide. The absorbance of the reaction mixture was immediately read at 590 nm against a blank containing PBS, potassium iodide and acetic acid. AOPP concentrations were expressed as micromoles per litre of chloramine-T equivalents.

Erythrocyte rheological response to reactive oxygen species (ROS) in human

In human blood samples, erythrocytes were incubated with either 5.4 $mmol \cdot l^{-1}$ of t-butyl hydroperoxide (TBHP, an oxidant) diluted in ethanol (Sigma Aldrich, St Quentin-Fallavier, France) (TBHP condition) or with ethanol alone (SHAM condition) for 10 min at 23°C. Erythrocytes treatment with TBHP 5.4$mmol \cdot l^{-1}$ for 10min at 23°C was chosen as it does not cause hemolysis [14]. At the end of the incubation period (TBHP and SHAM), the erythrocytes suspension was washed 3 times with phosphate buffer solution and re-suspended in autologous

plasma at a standard hematocrit of 40% prior to hemorheological measurements of erythrocyte deformability and aggregation.

Statistical Analyses

Data are presented as mean ± SD. All dependent variables were assessed for a normal distribution via the Shapiro-Wilk test and equal variance via the Brown-Forsythe test. Differences between the general characteristics between OSA patients and control participants, and rats exposed to IH and normoxia were determined using independent t-tests or Mann-Whitney Rank Sum tests depending on data distribution and equality of variance. As OSA may be protective or deleterious with respect to cardio- and cerebrovascular disease depending on disease severity [15], patients were divided into two groups based upon the median ODI - less severe (ODI < 35.4 events·h^{-1}) and more severe (ODI ≥ 35.4 events·h^{-1}). Differences between control participants, less severe OSA patients and more severe OSA patients were determined using a one-way analysis of variance (ANOVA) or Kruskal-Wallis one-way ANOVA on ranks while incorporating either a Tukey or Dunn's multiple comparison procedure, respectively. A chi-square analysis was used to assess differences in sex distribution.

To test the impact of oxygen and CPAP therapies on disease severity we compared post-oxygen and post CPAP with baseline data related to disease severity using a paired t-test or Wilcoxon's signed rank test depending on the distribution and equality of variance of the data. To test the impact of nocturnal oxygen therapy and CPAP treatment of OSA, on blood rheological parameters and redox balance, we performed a mixed factor 2x2 [group (controls/OSA)-by-visit (either pre- and post-oxygen or pre- and post-CPAP)] repeated measures ANOVA. For significant interaction effects, post hoc analyses incorporated a Tukey's correction for multiple comparisons. Alpha was set *a priori* at 0.05 and statistical analyses were performed with SPSS 23 (IBM SPSS Statistics, Chicago, IL, USA).

Figure E1. Experimental protocol

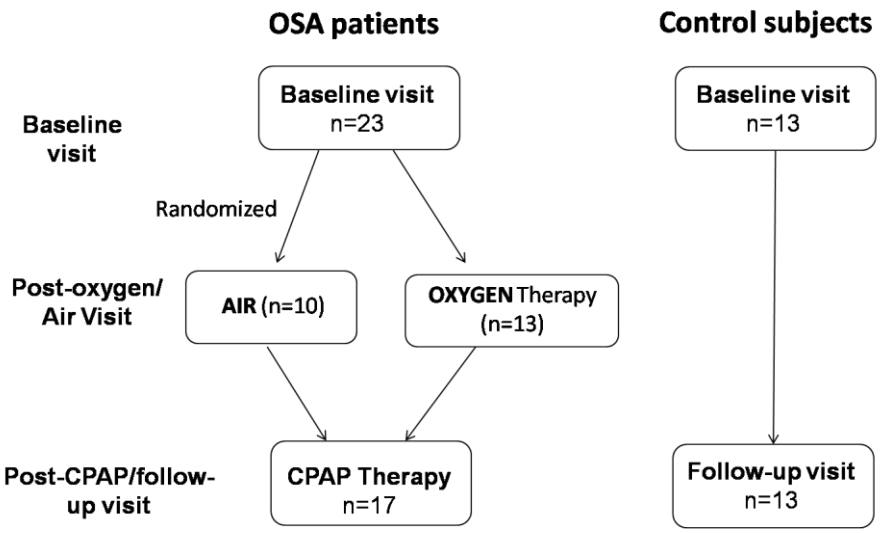


Figure E2. Effects of oxygen therapy on blood rheological parameters. OSA AIR: OSA patients not treated with oxygen (n=10); OSA O_2: OSA patients treated with oxygen for 2 weeks (n=12).

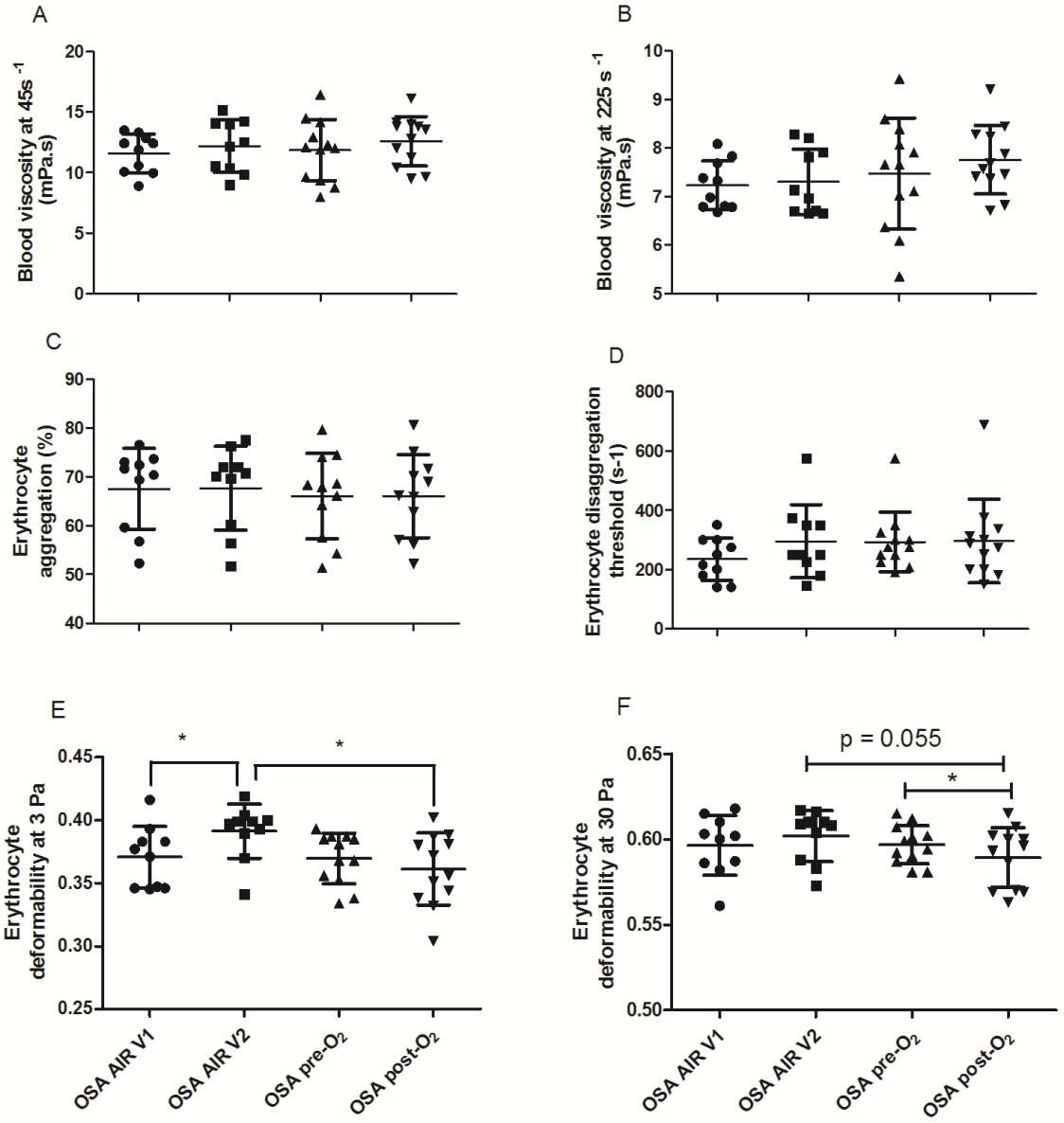


Figure E3. Effects of CPAP therapy on blood rheological parameters. Control participant n=13; OSA pre-CPAP n=17; OSA post-CPAP n=17.

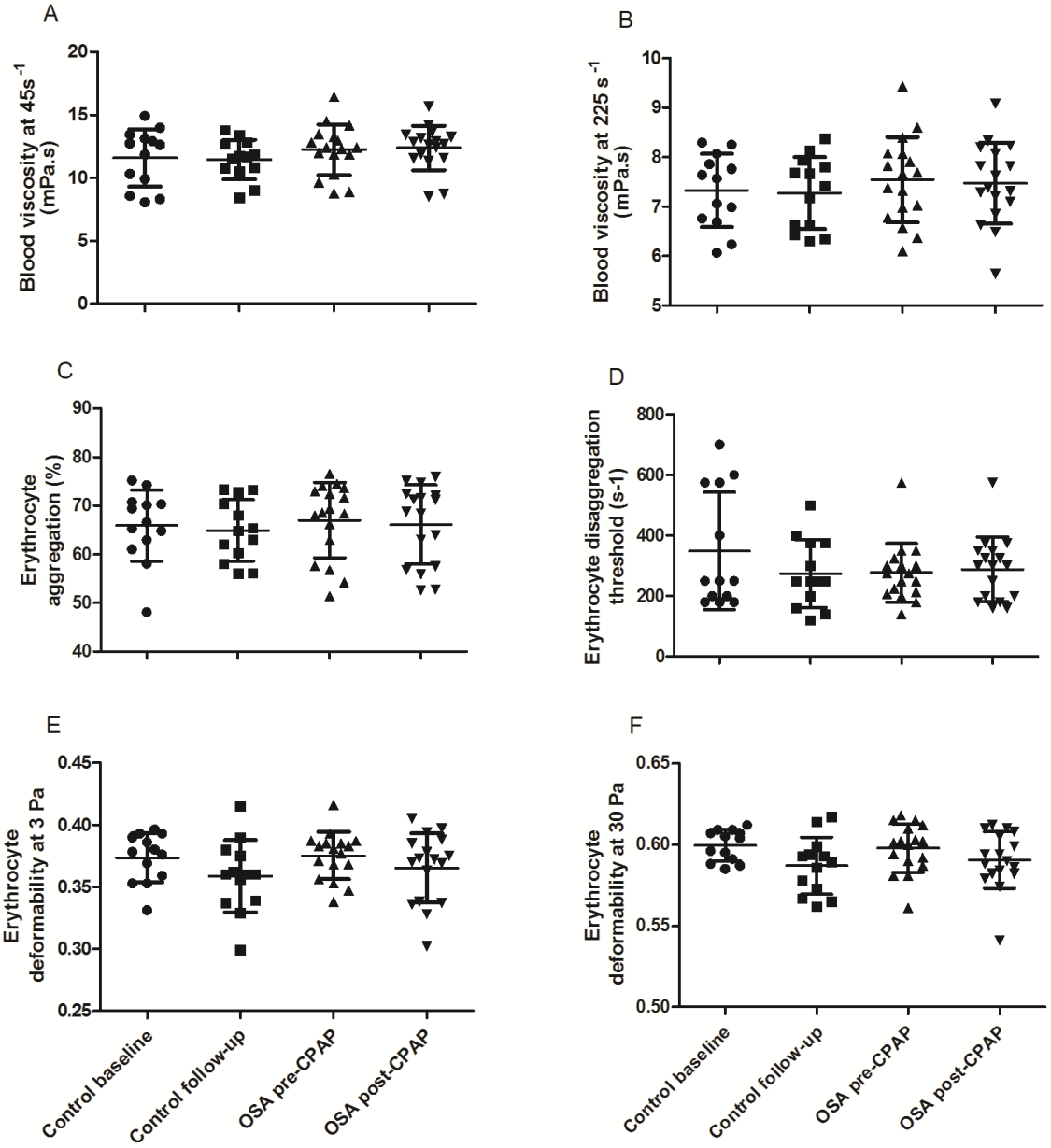


Figure E4. Effects of oxygen therapy on blood rheological susceptibility to reactive oxygen species (TBHP incubation). OSA AIR: OSA patients not treated with oxygen (n=6); OSA O_2: OSA patients treated with oxygen (n=7).

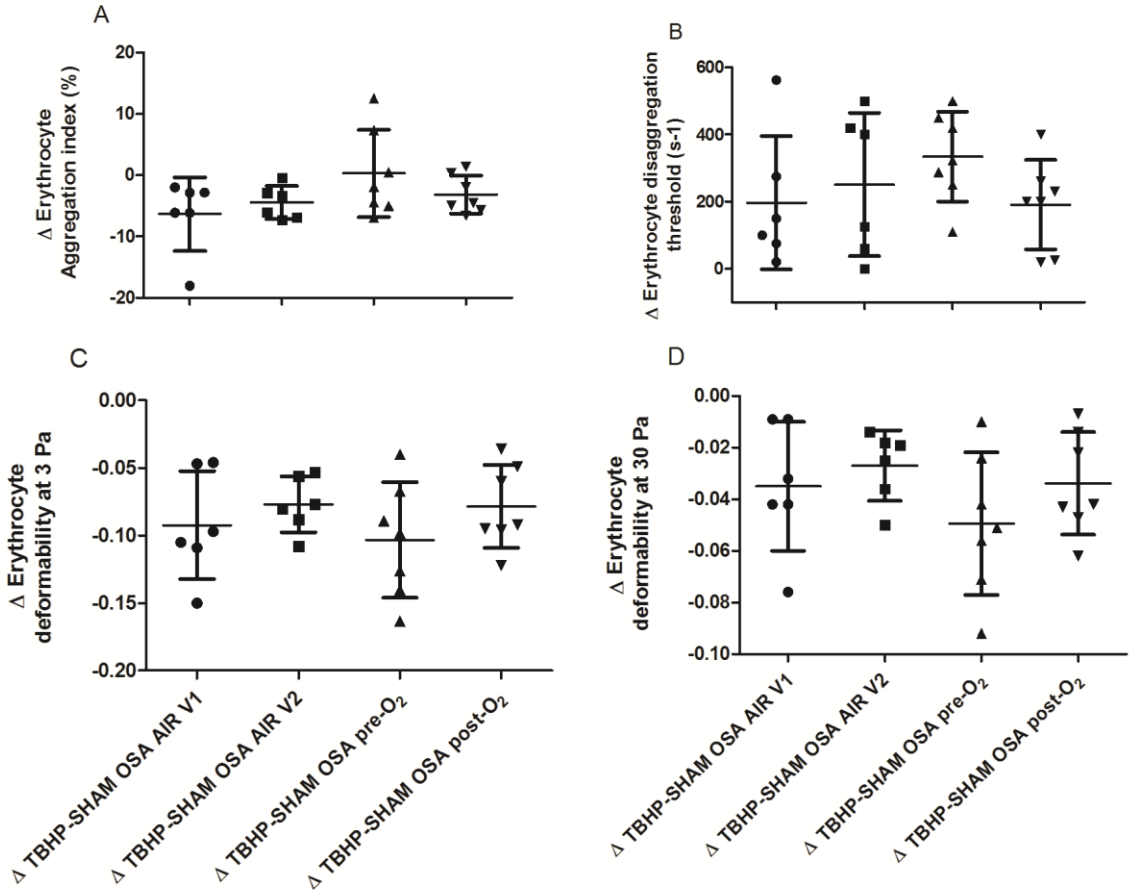
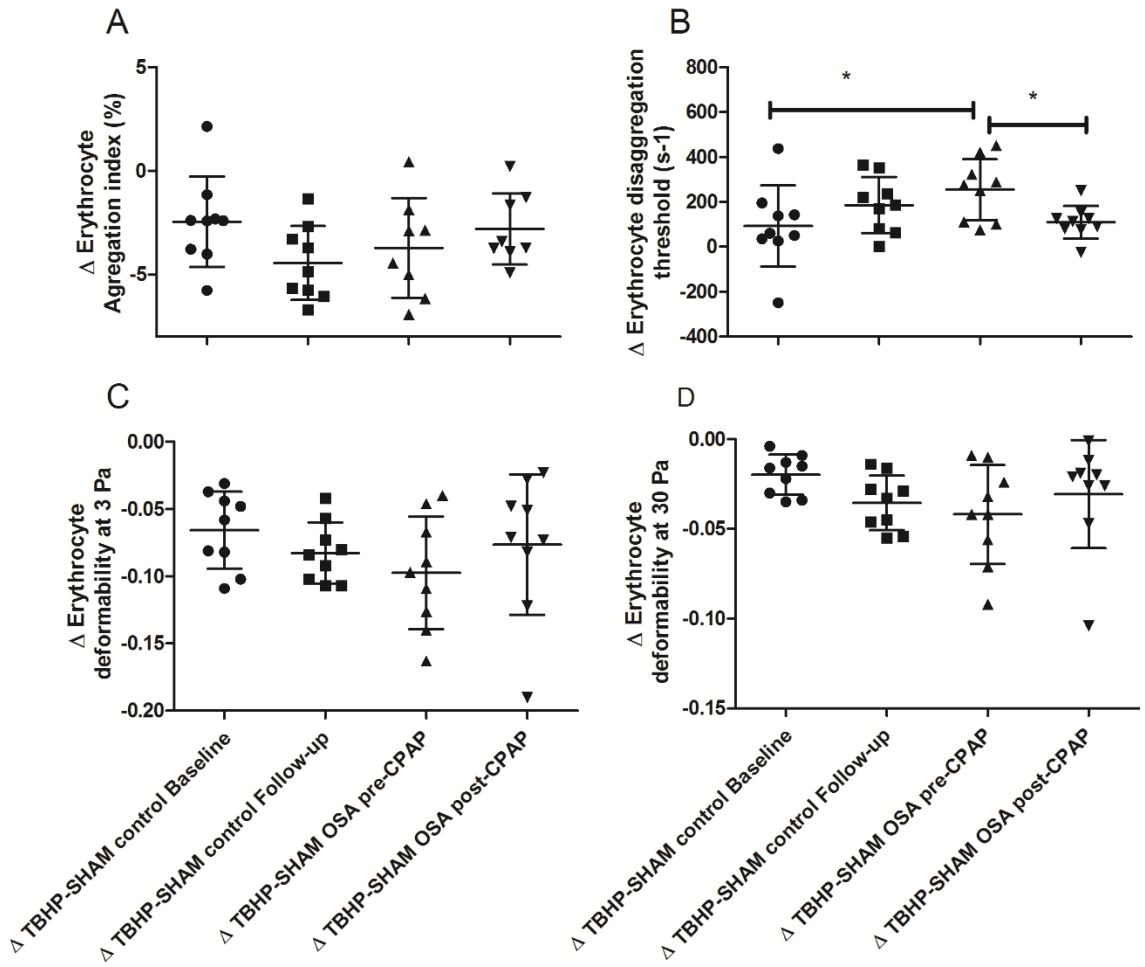


Figure E5. Effects of CPAP therapy on blood rheological susceptibility to reactive oxygen species (TBHP incubation). Control participant n=9; OSA patients n=9.



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