ONLINE DATA SUPPLEMENT

METHODS

Study participants

Newly diagnosed patients with severe OSA were consecutively recruited from the sleep unit at La Paz University Hospital-Cantoblanco, Madrid, Spain. Patients over 35 years of age and with an apnea-hypopnea index (AHI) of 30 or greater were included in the study. The diagnosis of OSA was established by respiratory polygraphy (Embletta GOLD, ResMed, San Diego, CA, USA), which included continuous recording of oronasal flow and pressure, heart rate, thoracic and abdominal respiratory movements, and oxygen saturation (SpO_2) (1, 2).Those tests in which the patients claimed to sleep less than 4 hours or in which there were less than 5 hours of nocturnal recording were repeated. Exclusion criteria were the following: previous history of thrombosis; previous or current treatment with oxygen or mechanical ventilation; patients who were underweight (body mass index [BMI] <18.5 Kg/m^2) or morbidly obese (BMI >40 Kg/m^2); history of respiratory, cardiovascular or metabolic disease, including chronic obstructive pulmonary disease, asthma, respiratory failure, hypertension, heart failure,orcoronary artery disease, diabetes or dyslipidemia; any infectious disease in the previous 3 months; diagnosis of malignancies or chronic inflammatory diseases; and the use of inhaled or systemic corticosteroids or other anti-inflammatory drugs. As a control group, healthy volunteers were selected who were homogeneous in sex, age and smoking habit. None of these volunteers were being treated with any type of medication, and the diagnosis of OSA was ruled out by respiratory polygraphy.

The study was approved by the local Ethics Committee (registry number PI-1857), and informed consent was obtained from all the participants.

Collection and preparation of samples

Peripheral blood samples from patients and controls were collected in 3.8% sodium citrate (Becton Dickinson, Madrid, Spain). All samples were collected between 9 a.m. and 11 a.m. Blood cell counts were performed with a Coulter AcT Diff cell counter (Beckman Coulter, Madrid, Spain).

Platelet-rich plasma (PRP) was obtained by whole blood centrifugation (150 g for 20 min at 23°C).

Platelet-free plasma (PFP) was obtained by centrifuging twice at 1800 g for 15 min at 23°C. Plasma aliquots were stored at −80°C until analysis. All the samples were analyzed or stored properly within 2 hours of sampling to preventthe release of cell microparticles (MPs) due to storage.

Rotational thromboelastometry

ROTEM (Pentapharm, Munich, Germany) is a viscoelastometric clotting test that evaluates the kinetics of clot formation and fibrinolysis. ROTEM was performed on whole blood properly recalcified by a non activated thromboelastometry test. ROTEM evaluates clotting time (CT: time from start of test until initiation of clotting, in seconds); clot formation time (CFT: time

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from CT to an amplitude of 20 mm, in seconds); α angle (tangent to the curve at 20 mm amplitude, in degrees); amplitude at "x" time (in mm); maximum clot firmness (MCF: reflects the strength of the thrombus, in mm); maximum velocity of clot formation (MAX$_V$), and lysis at 60 minutes (LI60, indicates residual clot firmness at 60 minutes, in %). These parameters are shown in Figure S1.
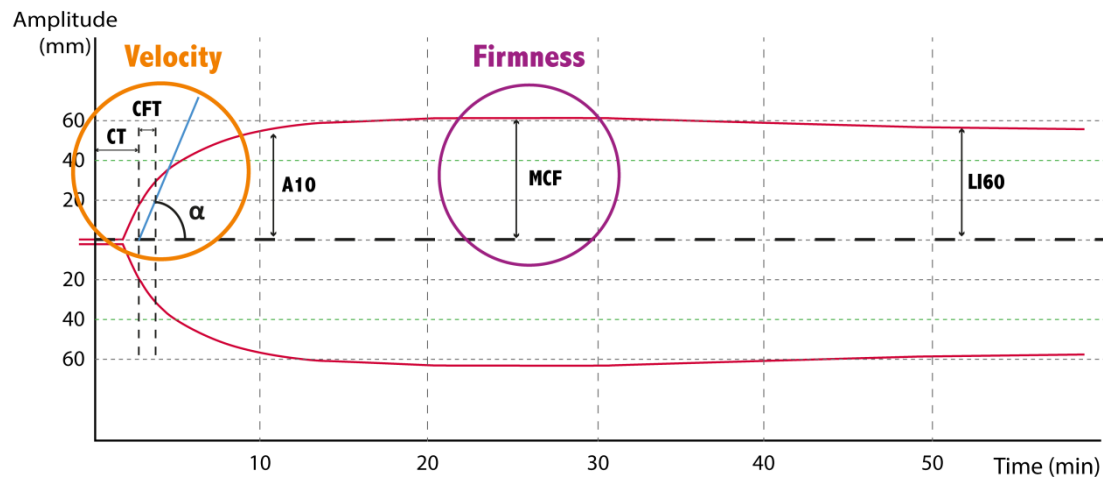

Figure S1. Representative plot of a ROTEM experiment indicating the measured parameters.

Determination of receptors and platelet activation markers

Platelet-rich plasma (PRP) was diluted 1:4 with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 5mM KCl, 145 mM NaCl, and 1 mM MgSO$_4$, pH 7.4) and incubated either without or with 100 µM thrombin receptor-activating peptide 6 (TRAP, Bachem, Switzerland) or 20 µM adenosine diphosphate (ADP, Sigma, Madrid, Spain) at room temperature (RT) for 15 min. Activated fibrinogen receptor, and exposure on platelet surface of P-selectin and of tetraspanin CD63 (which are usually contained, respectively, within the alpha and dense granules) were considered activation markers. They were evaluated through the binding of fluorescein (FITC)–PAC1 (Becton Dickinson, Madrid, Spain), a monoclonal antibody (mAb) that only recognizes the activation-induced conformational epitope PAC-1 on fibrinogen receptor, and FITC-labelled anti P-selectin mAb (BD Pharmingen, San Diego, California, United States) or FITC-anti-CD63 mAb (Becton Dickinson, Madrid, Spain). These antibodies were added for 15 min in dark at room temperature (RT). Surface expression of fibrinogen receptor was determined by labelling diluted PRP with Phycoerythrin (PE)-mAbs against its αIIb (BioCytex, Marseille, France) and β3 (Beckton Dickinson) subunits and von Willebrand factor receptor by labelling with FITC-labelled antibodies against GPIb and GPIX subunits of this receptor (BD Pharmingen, Madrid, Spain).

After 15 minutes, samples were diluted with phosphate-buffered saline (PBS) for analysis with a FACScan flow cytometer (Becton Dickinson, Madrid, Spain).

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Determination of leukocyte-platelet aggregates

To evaluate the formation of leukocyte-platelet aggregates, whole blood was diluted 1:10 in HEPES buffer and incubated with PE-αII mAb (Biocytex, Marseille, France) for the detection of platelets and the pan-leukocyte marker FITC-CD45 mAb (Becton Dickinson, Madrid, Spain). In platelet activation experiments, 50 µM TRAP or 40 µM ADP were added during fluorescent labelling of the blood samples for 15 min at RT in the dark. Samples were diluted with PBS for analysis by flow cytometry. Platelet-leukocyte aggregates were defined as leukocytes positive for PE-αIIb.

Calibrated automated thrombogram

Thrombin generation was assessed in PFP in fresh by calibrated automated thrombogram (CAT). Non-MP-associated plasma procoagulant activity was determined after proper recalcification and the addition of (final concentrations) 1 pM of recombinant human tissue factor and 4 µM of phospholipid mixture (PPP-Reagent LOW).

Procoagulant activity associated with MP content of either tissue factor (TF) or phosphatidylserine (PS) was determined in PFP by CAT using, respectively, MP-Reagent (4 µM of phospholipid mixture) and PRP-Reagent (1pM of recombinant human TF). All CAT reagents were from Diagnostica Stago (Spain). Samples were analyzed in duplicate. A Fluoroskan FL instrument (Thermo Labsystems, Helsinki, Finland) recorded the thrombin progress curves under the control of Thrombinoscope software version 3.6 (Thrombinoscope BV, Maastricht, Netherlands), filtered for excitation at 390 nm and emission at 460 nm. The lagtime (time from start of test until 10 nM thrombin was formed), the peak height of the curve (the maximum thrombin concentration generated); the time to reach the peak and endogenous thrombin potential (total amount of thrombin generated over time) were determined. These parameters are shown in Figure S2.
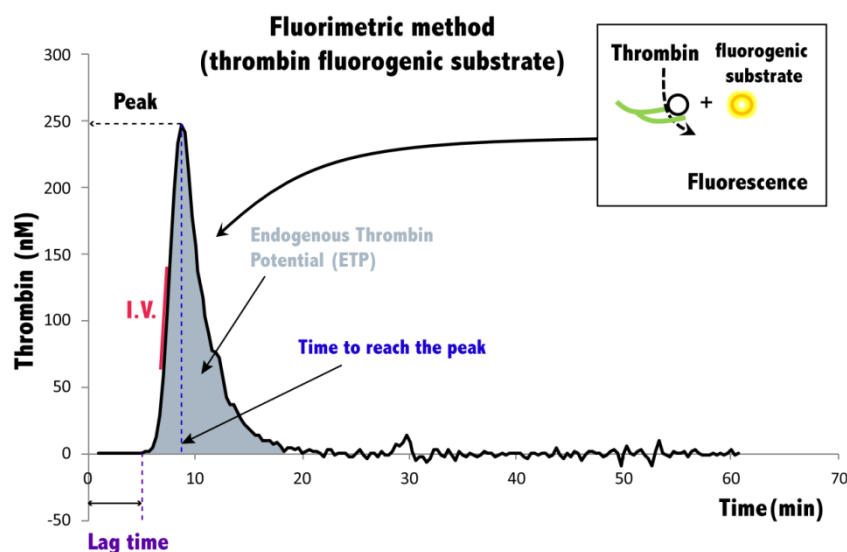

Figure S2. Representative plot of a CAT experiment indicating the measured parameters.

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Determination of E-selectin

Plasma levels of E-selectin were determined by enzyme-linked immunosorbent assay (ELISA) (Human sE-selectin/CD26E Immunoassay Quantikine ELISA kit, R&D Systems Europe Ltd., Abingdon, UK) in PFP, following the manufacturer's instructions.

Quantification of cell free DNA

Cell free DNA (cfDNA) was measured in PFP using the Quant-iTTMPicoGreen® dsDNA assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.
A standard curve from 1 ng/mL to 1 µg/mL with Lambda DNA was prepared. Then, 40 µL of sample were served into 96-well plate (Thermofisher) followed by adding 40 µL of working solution from the Quant-iTTMPicoGreen® reagent to each well. Plate was incubated for 5 minutes at RT. The fluorescence intensity was evaluated by a fluorescence lector (BioTek Synergy 4 Multi Detection Microplate Reader, BioTek Instruments, USA) and data was analysed by Gen5 software.

REFERENCES

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2-Kapur VK, Auckley DH, Chowdhuri S, Kuhlmann DC, Mehra R, Ramar K, Harrod CG. Clinical practice guideline for diagnostic testing for adult obstructive sleep apnea: an American Academy of Sleep Medicine clinical practice guideline. J. Clin. Sleep. Med. 2017; **13**: 479-504.