

Supplemental material

Methods

SM1: FOXP3 promoter methylation profile

Genomic DNA was isolated from blood samples using the Illustra blood genomicPrep Midi Flow kit (GE Healthcare, Little Chalfont, UK) following the recommended protocol. Approximately 1 g of genomic DNA was used for bisulfite modification using the EZ-96 DNA Methylation™ Kit (ZYMO, Irvine, CA) according to manufacturer instructions. After modification DNA was purified using ZR-96 DNA Clean-up Kit™ (ZYMO, Irvine, CA).

Primers designed were: FOXP3_METH_FW 5'
AAGAGAGAGGTTTGCGGTTTTTATATC 3',

FOXP3_METH_RV 5' AAAAAAATCAACCTAACTTATAAAAAACTATCACG
3',

FOXP3_UNMETH_FW 5'
TTATTAGAAGAGAGAGGTTTGTGGTTTTTATATTG 3'

and FOXP3_UNMETH_RV 5' CAACCTAACTTATAAAAAACTATCACATA 3'.

Reactions were performed in a 20 µl final volume using the MeltDoctor™ HRM master mix (ThermoFisher Scientific, Waltham, Massachusetts, USA), 0.6 µl of each primer (10 µM) and 1 µl of DNA (20 ng/µl) as template. Cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles consisting of a 15 second denaturation step at 95 °C, an annealing temperature at 60°C for 1 min and an extension step at 72 °C for 5 min. After amplification melt curves were run to collect data gradually increasing the incubation temperature from 65 °C to 90 °C at a rate of 0.1 °C/s and holding for 2 s after each stepwise increment. Melt curves were examined using the High Resolution Melting (HRM) Software V3.0.1. The standard curve to quantify methylation percentages were established at 100, 75, 50, 25, and 0% DNA methylation using the Epitect control DNA set (Qiagen, Venlo, Netherlands). Melting curves from methylated and unmethylated DNA

controls were normalized by selecting the “line of best fit” between 2 normalization regions selected before and after obtaining the raw data of the dissociation curve to generate a better profile of methylation percentages (1). The HRM analyses were performed as previously described (2). Methylation proportion in the samples was determined by comparing them with the standard curve generated by mixing different ratios of methylated and unmethylated DNA. As HRM standard curves with 0, 25, 50, 75 and 100% methylated DNA displayed uniform resolution, a percentage of methylation value was applied to every sample.

SM2: FOXP3 gene expression

For RNA extraction whole blood samples were collected in PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and frozen at -80°C . RNA was immediately isolated after thawing at room temperature using the PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Isolated RNA was reverse transcribed into complementary DNA (cDNA) using the SuperScript III System (Life Technologies, Inc.). The cDNA was diluted 1:20 for use in real-time PCR.

Two step TaqMan quantitative real time PCR (qRT-PCR) was performed to determine *FOXP3* expression in OSA patients and controls. *TRAP1* (TNF receptor-associated protein 1), *DECR1* (2,4-dienoyl CoA reductase 1, mitochondrial) and *PPIB* (Peptidylprolyl isomerase B) were used as housekeeping genes. These genes display stable expression in whole blood of humans of both genders with multiple disease conditions and ages (48). Predesigned TaqMan Gene Expression Assays (Applied Biosystems) for the test (assay ID: Hs01085834_m1 – *FOXP3*) and reference genes (assay ID: Hs00212476_m1 - *TRAP1*, Hs00154728_m1 - *DECR1*, Hs00168719_m1 - *PPIB*) were obtained from Applied Biosystems (Life Technologies Corporation, Carlsbad, CA, USA). Quantitative analyses were performed using StepOne and ViiA7 systems

(Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). A normalization factor (NF) was calculated as the geometric mean of the expression levels of the three housekeeping genes and it was used to normalize *FOXP3* mRNA expression levels. In addition three samples were repeatedly amplified in the different PCR plates to be used as IRC (inter-run calibrators) to remove run-to-run differences (49).

SM3: Data analysis

Logistic regression

In order to identify the association of the variables with each of defined categories (1: healthy, 2: sick, 1:healthy, 2: sick with low CPR,.....), we develop the following logistic regression:

$$\text{logit}(p_i) = \ln\left(\frac{p_i}{1-p_i}\right) = \mu + b_a a_i + b_t t_i$$

where p_i is the probability of belonging to the first category of the i th individual. μ is the general mean, a_i and t_i are the age in years and the measure of the clinical parameters for the i th individual, and b_a and b_t were the slope of the logistic regression associated with age and the clinical parameters. Statistical significance for the b_t parameter was calculated using the R package (R Core team, 2008)

Log-linear model

Given the non-gaussian nature of *FOXP3* expression and other data, we used a log-linear model to identify relationships between *FOXP3* expression levels and clinical parameters by using the model:

$$\log(y_i) = \mu + b_a a_i + b_t t_i + e_i$$

where y_i , a_i and t_i were the *FOXP3* expression level, the age in years and measure of the clinical parameter for the i th individual, respectively. Further b_a and b_t were the slope of the log-linear regression analysis related with age and the clinical

parameters, and e_i is the residual for i th individual. Statistical significance for the b_t parameter was calculated using the R package (R core team, 2008).

Supplementary Figures and Table

Table S1. Selection criteria in the EPIOSA study.

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> • Adults aged 20 – 60 years • AHI \geq 10 events per hour of sleep (OSA group) and AHI $<$ 5 (control group) • Willingness to participate in the study and complying with the study by signing a written informed consent • Available for study visits over 5 years 	<ul style="list-style-type: none"> • Any history of cigarette/tobacco smoking use • Alcohol abuse • Arterial blood hypertension (arterial blood pressure: \geq140 mmHg systolic and/or \geq90 mmHg diastolic or taking antihypertensive medication) Low High-density lipoprotein (HDL) cholesterol ($<$40 mg/dL) or high LDL cholesterol (\geq130 mg/ dL) or taking lower lipid drugs. • Fasting plasma glucose $>$ 126 mg/dl or taking anti diabetic medications • Other metabolic diseases (e.g., hypothyroidism) • Present or past history of vascular diseases, including myocardial infarction, angina, coronary artery procedures (coronary artery bypass graft or percutaneous coronary intervention), aneurysm, transient ischemic attack or stroke. • Autoimmune diseases • Past or present history of malignancies • Chronic inflammatory diseases (e.g., Crohn disease) • Chronic infectious diseases (e.g., chronic viral hepatitis) • Chronic respiratory diseases (e.g., asthma) • Morbid obesity (body mass index \geq 40 kg/m²) • Any chronic oral therapy

- Sleep disorders other than OSA
- Having undergone upper airway surgery
- Having previously use CPAP therapy

OSA = Obstructive sleep apnea, AHI = apnea-hipopnea index, CPAP = continuous positive airway pressure.

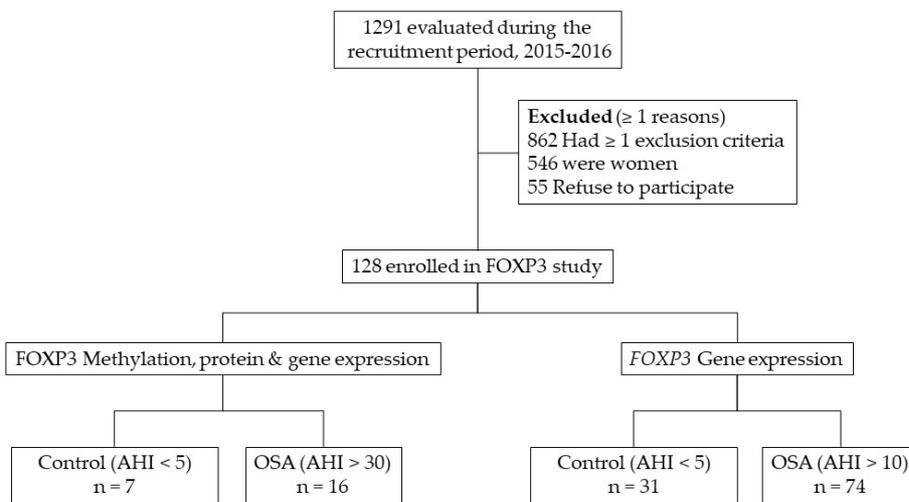


Figure S1. Study Flow Diagram

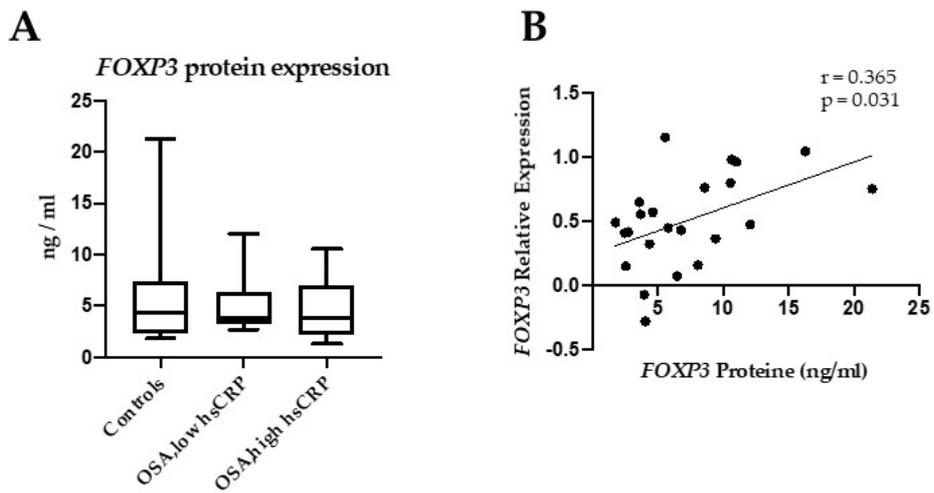


Figure S2. FOXP3 protein expression (A) and its relationship with gene mRNA expression.

Supplemental References

1. Wojdacz TK, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic acids research* 2007; 35: e41.
2. Wojdacz TK, Dobrovic A. Melting curve assays for DNA methylation analysis. *Methods in molecular biology* 2009; 507: 229-240.

