Official Title: **Epigenetics Modifications in Morbid Obesity and Obstructive Sleep Apnea Patients (EPIMOOSA): CPAP and Bariatric Surgery Impact. EPIMOOSA Study**

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Unique Protocol ID: 23/2014

Study design. This is a 2-year prospective, longitudinal, noninterventional cohort study. It will be conducted in the Sleep-Disordered Breathing Units at the Miguel Servet University and Royo Villanova hospitals in Zaragoza.

Participant selection and follow-up. Patients will be selected from the bariatric surgery (BS) waiting list based on the inclusion criteria set out in Table 1. At the first visit, which will include a polysomnography, the study team will decide whether to begin continuous positive airway pressure (CPAP) therapy in accordance with Spanish standards for the treatment of obstructive sleep apnea/hypopnea syndrome (OSAHS) (1). All participants will be asked to attend a 6-month follow-up visit in which adherence to CPAP therapy (if applicable) will be assessed and a date for BS will be set, if the procedure is still recommended. Patients will then attend 3, 6 and 12-month postoperative follow-up visits.

INCLUSION CRITERIA	EXCLUSION CRITERIA
Age 18–60 years	Obesity hypoventilation syndrome or treatment with positive pressure devices

BMI consistently > 40 kg/m^2 for 3–5 years, following more than 1 year of unsuccessful controlled medical treatment	Diagnosis of systemic inflammatory disease
BMI 35–40 kg/m^2 with comorbidities associated with MO susceptible to improvement with weight loss (high BP, DM, dyslipidemia, OSA, etc.), following more than 1 year of unsuccessful controlled medical treatment	Neoplastic diseases in the last 5 years
A signed informed consent form	A cardiovascular event in the last 6 months
	Pregnancy

Tabla 1. Selection criteria

The control group will comprise subjects from the EPIOSA study that is currently underway (2). EPIOSA is an observational cohort study conducted by the Miguel Servet University Hospital in Zaragoza that aims to investigate the natural history of OSA in relation to potential epigenetic changes. Patients with and without OSA, adjusted for sex and age ($\pm$ 2 years), will be selected from the EPIOSA study population, which excludes individuals with a BMI > 35 kg/m^2, for comparison with patients in the MOOSA study.

Sample size.

The aim is to form three patient groups: a) subjects with MO and without OSA; b) patients with MO and OSA who do not need CPAP therapy; and c) patients with MO and OSA requiring CPAP therapy. Each subgroup must include at least 12 subjects who complete the 2-year follow-up after their inclusion in the baseline visit. The sample size has been calculated to reveal significant differences in the epigenetic changes between the groups with OSA and the control group (main objective). In a previous study, Kim et al. (3) already demonstrated significant differences in DNA methylation of proinflammatory genes among two subgroups of eight children with OSAHS with high- or low CRP. In our cohort, we observed significant differences in circulating exosome levels between groups of 12 patients with OSA versus controls. Therefore, considering a potential loss to follow-up of 10%, we proposed the inclusion of 15 subjects per subgroup analyzable after a minimum 2-year follow-up, which equates to a final sample size of 45 patients.

These 45 patients will subsequently be compared with another 45 cases from the EPIOSA study. Therefore, 90 patients in total will be included: 45 from the EPIMOOSA study and 45 from the EPIOSA study.

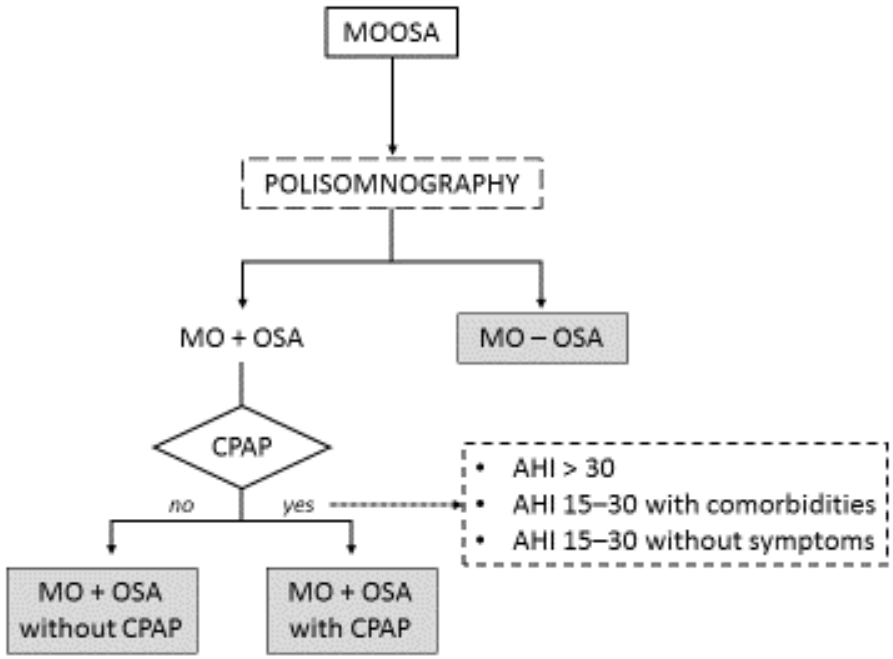

Figura 1. Protocol Design

Table 2 shows the schedule for collecting data on each variable during the various study visits.

VARIABLE	V0	V1	V2	SURGERY	V3	V4	V5
Time	Inclusion	3m	6 m	12 m	15 m	18 m	24 m
Clinical History	*	-	*	-	*	*	*
Anthropometry	*	-	*	-	*	*	*
Polysomnography	*	-	-	-	-	-	*
Blood test	*	-	*	-	*	*	*
Arterial Blood Gas	*	-	-	-	-	-	*
AutoCPAP	-	*	-	-	-	-	-
miRNA	*	-	*	-	*	*	*
Electrocardiogram Holter	*	-	*	-	*	*	*
Blood pressure Holter	*	-	*	-	*	*	*

Tabla 2. Study Variables

***Clinical data*.** The following clinical data and complementary tests will be recorded at each visit: a) sociodemographic data, clinical, surgical and family history,

and regular medications; b) level of daytime sleepiness based on the Epworth scale (4); c) weight (kg), height (cm), body mass index (BMI = weight (kg)/height $(m)^2$), and neck, waist, and hip circumferences (all in cm); d) blood pressure, measured according to the European Society of Hypertension (ESH) and the European Society of Cardiology (5) clinical practice guidelines (6); and e) spirometry, measured in line with European Respiratory Society (ERS) standards (7).

***Sleep studies*.** We will use a validated home polysomnography system (ApneaLink vs10.20 ResMed®). The process consists of a continuous recording of airflow using a nasal cannula, chest movement, oxygen saturation, snoring, and body position. We define apnea as a lack of airflow for more than 10 seconds, hypopnea as the reduction of airflow (> 50%) for over 10 seconds accompanied by a decrease in oxygen levels of greater than 4%, and the apnea–hypopnea index (AHI) is expressed as the sum of instances of apnea and hypopnea per hour over the period studied. The principal investigator (PI) will interpret the results of the polysomnography.

Each patient will be administered CPAP therapy or not based on recommendations from the Spanish Society of Pulmonology and Thoracic Surgery (SEPAR, its acronym in Spanish). This decision will be made by the PI on a patient-by-patient basis according to severity of OSAHS, cardiovascular comorbidity, and/or daytime symptomatology. Patients treated with CPAP therapy will receive an extra visit to carry out a pressure titration at home using AutoCPAP (AutoSet ResMed®) (8).

Blood pressure and electrocardiogram (ECG) Holter. All patients will complete a 24-hour blood pressure and electrocardiogram study. This shall be performed using a Labtech®EC-3H/ABP that combines a 3-channel ECG Holter and an ambulatory blood

pressure monitor. These studies will be conducted the day after the home polysomnographies during the first and final visits, and over the 24-hour period before collecting blood samples for all other visits. They will interpreted according to the guidelines published by the Spanish Society of Cardiology (9).

***Blood tests*.** Blood samples will be collected at each visit. Venous blood samples will be obtained with 21G Abbocaths and arterial samples with 23G ProVent® kits for blood gas analysis. Glucose, triglycerides, total cholesterol, HDL, LDL, and apolipoprotein A and B blood levels will be analyzed by spectrophotometry (IMMAGE® 800 Protein Chemistry Analyzer, Beckman Coulter). High-sensitivity CRP will be determined within 2 hours of collecting blood samples using flow nephelometry. 15 mL of blood will be used to obtain serum and plasma, and 5 mL with EDTA to conduct genetic and epigenetic studies; these samples will be stored in a freezer at -80 °C until they are analyzed.

Exosome and microRNA analysis. Circulating exosomes and the encapsulated microRNA will be studied at the Translational Unit of the Miguel Servet Hospital in Zaragoza. Blood samples will be collected at each visit and frozen at -80 °C on the premises of the Royo Villanova Hospital. Once patient enrollment is complete, the blood samples will be transferred using containers filled with dry ice to the Translational Research Unit at the Miguel Servet Hospital. MicroRNA will be obtained using the PAXgene Blood miRNA Kit (PreAnaltiX) following the manufacturer's protocol. RNA integrity will be ensured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). After extracting the RNA samples and ensuring their integrity, they will be reverse transcribed using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, California, USA). Mature miRNA will

then be quantified by means of real-time CRP (TaqMan® MicroRNA Assays). Finally, miRNA expression shall be analyzed using BioMark 96.96 Dynamic Array™ (Fluidigm Corporation, San Francisco, California, USA). The results will be expressed following the method $2-\Delta\Delta Ct$ (10)..Table 3 shows the miRNA that will be studied in the MOOSA study.

PANEL OF miRNA TO BE STUDIED IN THE MOOSA PROTOCOL		
UniSP2	miR-320a	miR-16-5p
UniSP5	miR-145-5p	miR-126-3p
cel-miR-39	miR-146A-5p	miR-133a-3p
let 7a-5p	miR-223-3p	miR-34a-5p
miR-21-5p	miR-155-5p	

Tabla 3. Panel of miRNA to be studied in the EPIMOOSA protocol.

Schedule. The study has already been initiated thanks to support from SEPAR and will overlap with the Bariatric Surgery Unit's care strategy. Patients are being enrolled from the waiting list for BS. The recruitment period will last for 2 years, while follow-up will run for 6 months after the polysomnography and 1 year after surgery. As seen in Figure 2, we plan to conduct six visits over a 2-year period, excluding the surgery during which no samples will be collected.

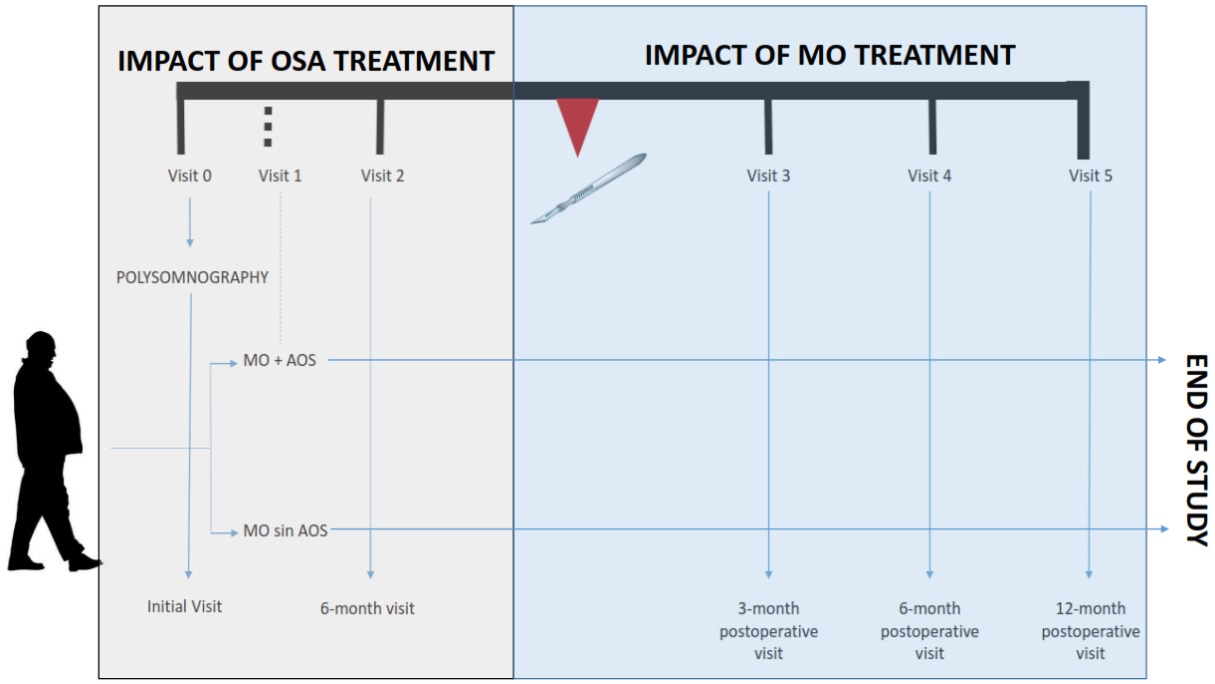

Figure 2. The study schedule includes two clearly distinct stages. The first stage features patients who have not yet undergone surgery for MO but who are being treated for OSA based on the initial polysomnography. Three months after the initial visit, patients with OSA who are receiving CPAP therapy will complete an AutoCPAP pressure titration. This will provide information on the impact of OSA treatment in patients with MO. The second stage will start 3 months after bariatric surgery. This phase involves a 12-month postoperative follow-up period with three visits, the last conducted 12 months after the BS because this is when weight loss is thought to stabilize. We shall study the impact of BS throughout this second follow-up period.

***Intervention allocation*:** CPAP therapy will be decided according to the PI's clinical judgment in line with SEPAR's clinical practice guidelines (1).

Statistical analysis: We have predefined the following statistical procedures: a) Description of populations. They will be expressed as mean values ± SD when variables are continuous. Frequencies distribution will be obtained for qualitative variables. b) Difference between groups: parametric Student's t-test or ANOVA—if they follow a normal distribution—or Mann–Whitney U-test or Kruskal–Wallis H-test—if we do not work with said hypothesis. For the qualitative variables, we will use the normal chi-

square test with standardized residuals. c) Relationship between systemic inflammation and clinical variables: Pearson's or Spearman's correlation depending on the variables' normality. d) Predictors of systemic inflammation and subclinical atherosclerosis: stepwise logistic regression. e) Effect of treatment: paired samples t-test, if we assume normality, or the Wilcoxon nonparametric test for variables that do not follow a normal distribution. f) GOstats software will be used to analyze epigenetic markers (Falcon S et al., Bioinformatics 2007;23:257). g) The interindividual variability of miRNA will be determined through the coefficient of variation. We will use GraphPad Prism 6 (GraphPad Software) and SPSS version 23.0 (IBM) statistical packages.

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