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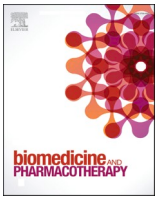

# Proteomic profiling for prediction of recurrent cardiovascular event in patients with acute coronary syndrome and obstructive sleep apnea: A post-hoc analysis from the ISAACC study

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## ABSTRACT

*Background:* Obstructive sleep apnea (OSA) is associated with a recurrent cardiovascular event (CVE) risk in patients with a first acute coronary syndrome (ACS). However, the pathological pathways by which OSA promotes this deleterious role are unknown. We aim to explore the proteomic profile associated with OSA that promote the recurrent CVE risk in severe OSA patients with ACS without previous cardiovascular diseases.
*Methods:* This post-hoc analysis from the ISAACC study (NCT01335087) included 86 patients admitted for ACS. Patients underwent respiratory polygraphy for the first 24–72 h to OSA diagnosis. We analyzed of 276 cardiovascular and inflammatory related proteins in baseline fasting plasma samples using proximity expression assay technology (Olink®, Sweden). Protein levels were compared between severe OSA patients with/without recurrent CVEs during follow-up. Random forest was conducted to select relevant proteins and generate a predictive model of recurrent CVE.
*Results:* We included 86 patients (median age: 61 years, median BMI: 29.4 kg/m$^2$ and 86 % males) admitted for ACS with severe OSA (56 without recurrent CVE/30 with recurrent CVE). The plasma levels of 38 proteins were

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differentially expressed between groups. Additionally, 12 proteins had a significant association with respiratory polygraphy parameters. Three proteins discriminate with an AUC of 0.81 (95 % CI of 0.71–0.9) between severe OSA patients with and without recurrent CVE. These proteins were implicated in cell proliferation, communication and apoptosis, and regulation/response to the inflammatory and immune systems.
*Conclusion:* In ACS patients with severe OSA, a proteomic profile was associated with recurrent CVEs. This proteomic profile was correlated with specific OSA parameters from respiratory polygraphy. Proteomic profiling may provide an new direction for patient risk stratification and clinical management.

## 1. Introduction

Obstructive sleep apnea syndrome (OSA) is a common chronic disease that affects 20–30 % of the adult population [1]. OSA is caused by the appearance of repeated episodes of obstructive apneas and hypopneas that occur during sleep. OSA imposes important pathophysiological consequences that lead to impaired quality of life, increased traffic and occupational accidents, depression, cognitive impairment, cardiovascular and metabolic diseases and, more recently, cancer [2–5].

Epidemiological and clinical data, together with basic research support that OSA has a role in the initiation or progression of several cardiovascular diseases (CVDs) [6]. Available data, mainly from observational studies, have suggested that OSA is an independent risk factor for cardiovascular morbidity and mortality [7] and it has been reported that a high proportion (40–60 %) of patients with CVD have OSA [8]. Acute coronary syndrome (ACS) is often the first manifestation of CVD and a major cause of morbidity and mortality that affects 1 % of the adult population worldwide [9,10]. It is estimated that from 50 % to 70 % of patients admitted for ACS have undiagnosed OSA [2,11]. The results from a recent study [12] suggest the existence of specific endotypes of patients with ACS in which OSA is associated with an increased risk of recurrent cardiovascular events (CVEs), concluding that in patients with a first ACS without previous CVD, OSA would be associated with an increased risk of recurrent CVE. Even in this endotype of ACS patients without previous CVD, the deleterious effect of OSA is heterogeneous since only part of these OSA patients suffered a recurrent event during follow-up. However, the mechanisms by which OSA would induce an increased risk of recurrence in these patients and the biomarkers associated with an increased risk of recurrence are still unknown.

In the present study, we aimed to identify the proteomic profile associated with the risk of recurrence of CVEs in patients with OSA admitted for a first ACS. The identification of this profile would also contribute to elucidating the specific physiopathological mechanisms associated with OSA that would contribute to the risk of recurrence of CVEs in patients with a first ACS without previous CVD.

## 2. Methods

### 2.1. Study design

This is an ancillary study of the ISAACC study (Continuous positive airway pressure (CPAP) for prevention of CVEs in ACS and OSA patients), a multicenter, open-label, parallel, prospective, randomized, controlled trial (trial registration number: NCT01335087) [13]. Full details of the ISAACC study's methods, including the inclusion and exclusion criteria have been previously reported [14]. Briefly, eligible patients were aged $\geq$ 18 years, had been admitted for ACS to coronary care units or cardiology hospitalization wards at 15 hospitals in Spain and had an Epworth Sleepiness Scale score of $\leq$ 10 (i.e., patients without excessive daytime sleepiness or nonsleepiness). Patients underwent respiratory polygraphy (Embletta, ResMed, Bella Vista, NSW, Australia) during the first 24–72 h after admission to evaluate the presence of OSA [14]. For the current post-hoc study, to identify the proteomic profile associated with the risk of recurrence of CVEs in patients with OSA admitted for a first ACS, we analyzed the data from 86 patients admitted for a first ACS, without previous CVD and with severe OSA (AHI$\geq$30 events/h) (Fig. 1). Additionally, as a reference group, we included 90 non-OSA patients to confirm the specific relationship between OSA and the proteomic profile of the risk of recurrence of CVEs.

ACS was defined as the acute presentation of coronary disease with or without Q-wave infarction (patients with a normal ECG and ischemic symptoms but only a minor rise and fall in any biomarker were included), unstable angina, or type 1 myocardial infarction [15]. Finally, to predict and stratify mortality risk of ACS, the Killip classification was used, referring to class I as less severity and mortality risk increasing to class IV.

### 2.2. Procedures and outcomes

Questionnaires to record demographic and anthropometric characteristics, medical history and usual pharmacological treatment were administered the day before the sleep study along with questionnaires associated with the degree of daytime sleepiness (Epworth Sleepiness Scale). All patients were evaluated at baseline and one month, three months, six months, 12 months, 18 months, 24 months, 30 months, 36 months and annually thereafter. All patients were monitored and followed up for a minimum of one year. At each visit, sociodemographic and anthropometric variables previously related to increased cardiovascular risk were recorded. Each follow-up visit included assessments of a composite of CVEs (cardiovascular death or nonfatal events [acute myocardial infarction, nonfatal stroke, hospital admission for heart failure, and new hospitalizations for unstable angina or transient ischemic attack]).

### 2.3. Proteomic analyses

Overnight fasting venous blood samples were obtained at baseline. Whole blood samples collected in EDTA (ethylene diamine tetraacetic acid) anticoagulant tubes were centrifuged at 1500 x *g* for 10 min at 4 °C to separate the plasma fraction. All specimens were immediately aliquoted, frozen, and stored in a dedicated −80 °C freezer. A total of 276 protein biomarkers were analyzed in baseline fasting plasma samples using the Olink® Target Cardiovascular II, Cardiovascular III and Inflammation panels (Olink Proteomics, Uppsala, Sweden) which comprised 92 proteins each and uses the methodology based on the proximity extension assay (PEA). These panels were selected for their known associations with the mechanical pathways involved in CVD and inflammation. Ten proteins were present in two panels, leaving 266 unique proteins in total. Plasma samples from selected patients from the ISAACC cohort were thawed, transferred to 96-well plates and shipped frozen on dry ice. All samples were randomized across plates and assays were performed in a blinded fashion. Data quality was controlled and normalized using an internal extension and an interpolate control to adjust for intra- and interrun variation.

Data are expressed as normalized protein expression (NPX) values. Proteins with less than 25 % of the values below the limit of detection (LOD) were included in the analyses.

### 2.4. Pathway enrichment analysis

Protein–protein interaction networks were studied using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database

with an interaction score of 0.700 (high confidence) [16]. For the analysis of functional pathways, the STRING and Reactome databases were used [17]. The protein set associated with diffusion capacity was used for both analyses. The false discovery rate (FDR) was calculated using the Benjamini—Hochberg procedure and set at <0.05. Cell, tissue and organ signatures were defined using data from the Genotype-Tissue Expression (GTEx) Portal (https://www.gtexportal.org).

### 2.5. Statistical analysis

The main characteristics were described using the median [25th percentile, 75th percentile] or n (%), and differences between groups were assessed using the Mann–Whitney U test for continuous variables or Fisher's exact test for categorical variables.

To evaluate differences in protein levels across the groups (no recurrent CVE and recurrent CVE), linear models and empirical Bayes methods [18] were used. A volcano plot was generated, and proteins with p value less than 0.05 were highlighted as proteins with significant differences in plasma levels between groups. The FDR was performed by the p value corrected for multiple testing using the Benjamini—Hochberg procedure. Furthermore, candidate proteins identified in the previous step also need to show a dose—response association with the respiratory polygraphy parameters. Multiple linear regression models between protein levels (treated as an outcome) and respiratory polygraphy parameters after adjusting for confounders (age, sex and body mass index (BMI)) were carried out. Protein levels, respiratory polygraphy parameters and confounding factors were previously standardized.

A Voronoi diagram was used to represent and clearly summarize the candidate proteins, the ones with significant differential expression between groups and also associated with the respiratory polygraphy parameters.

To analyze the relationship between the candidate proteins, a hierarchical clustering analysis was performed, including a dendrogram illustrating the clustering of proteins and samples.

Finally, the random forest algorithm was used as a classification technique to identify the key proteins for recurrent CVE prediction. Additionally, receiver operating characteristic (ROC) curve analysis was used to examine the classification accuracy of the key proteins to estimate the risk of recurrent CVE using a logistic regression model. Area under the curve (AUC) value together with 95 % confidence interval (95 % CI) are reported.

Statistical significance was set at p value <0.05. Data were analyzed using R, version 4.0 [19].

## 3. Results

### 3.1. Patient characteristics

From a total of 1851 patients with ACS enrolled in the ISAACC cohort, we included 209 patients with a first ACS without CVD diagnosed with severe OSA (AHI≥30 events/h) and with usual care treatment. Specifically, a total of 30 patients suffered a recurrent CVE during the follow-up and were included in the present study. Finally, we included 56 patients without CVEs with a minimum of 2 years of follow-up [20,21] (2:1) matched by sex, age and BMI (Fig. 1). The median age of the patients was 61 years, the median BMI was 29.4 kg/m$^2$ and 86 % were males. According to the Killip class and the number of affected vessels and stents placed, both groups of patients suffered a first CVE with similar severity, (Table 1). Moreover, both groups received similar pharmacological prescriptions at hospital discharge (Table A1). The patients were followed for a median of 3.25 (interquartile range (IQR): 2.88) years. The follow-up time was lower in patients with recurrent CVE. As indicated in the ISAACC study protocol patients were followed up until the development of a recurrent CVE.

The non-OSA reference group comprised 90 non-OSA patients from the ISAACC study. A total of 28 patients suffered a recurrent CVE during the follow-up (Fig. 1). The median age was 60.6 years and 85.5% were males. The median time of follow-up (or up to CVE) was 3.90 (IQR: 3.45) years (Table A2).

### 3.2. Quality control

All samples passed quality control. There were 10 proteins analyzed in two of the panels studied, and a high correlation between them was observed (Fig. A.1). Twenty proteins with more than 25 % of the values below the LOD were excluded from the analyses. Finally, 246 proteins were included in the analysis.

### 3.3. Identification of differentially expressed proteins in OSA patients with and without recurrent cardiovascular event

A total of 38 proteins were differentially expressed ($p < 0.05$) in severe OSA patients who suffered recurrent CVEs (Fig. A.2A and

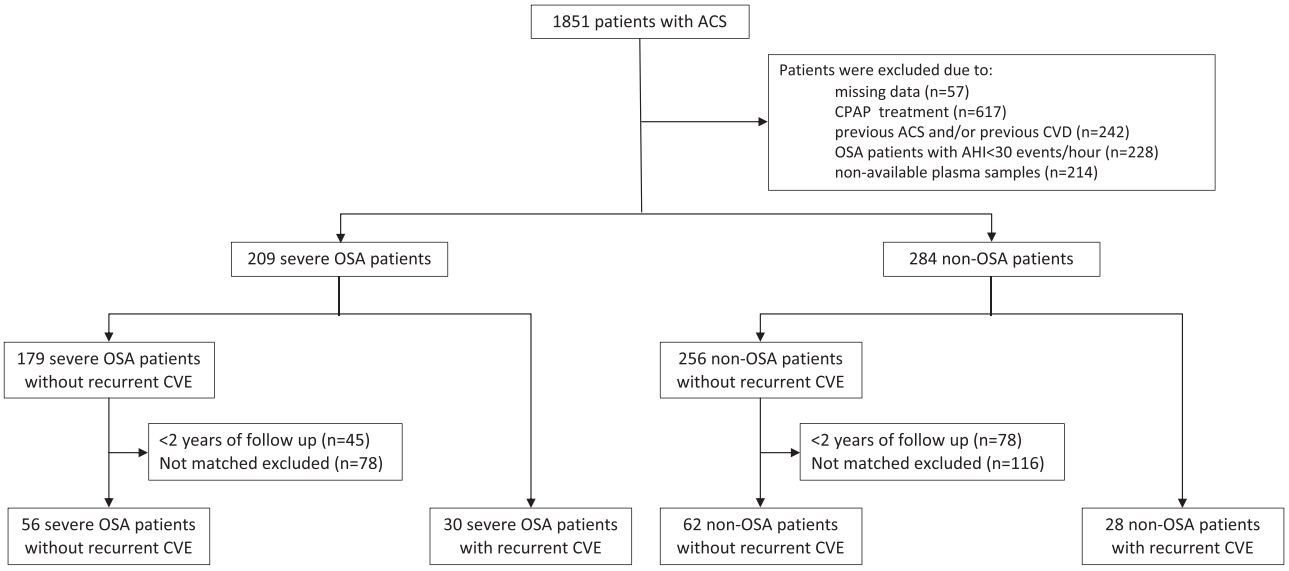


**Fig. 1.** Flowchart of the study.

**Table 1**
Baseline characteristics of included patients with OSA and with or without recurrent cardiovascular events.

| | OSA patients with recurrent CVEs (N = 30) | OSA patients without recurrent CVEs (N = 56) | p value |
|---|---|---|---|
| **Sex** | | | 0.101 |
| Female | 7 (23.3 %) | 5 (8.93 %) | |
| Male | 23 (76.7 %) | 51 (91.1 %) | |
| **Anthropometric measures** | | | |
| Age, years | 61.0 [53.2, 67.0] | 61.0 [56.0, 67.8] | 0.647 |
| Body mass index, kg/m$^2$ | 30.1 [26.4, 33.2] | 29.4 [26.2, 31.4] | 0.214 |
| Waist-hip ratio | 0.99 [0.95, 1.03] | 0.99 [0.96, 1.02] | >0.900 |
| Neck circumference, cm | 41.0 [40.0, 42.0] | 41.0 [38.5, 44.0] | 0.821 |
| **Lifestyle risk factors** | | | |
| Smoking | | | 0.935 |
| Never | 7 (23.3 %) | 13 (23.2 %) | |
| Former | 7 (23.3 %) | 15 (26.8 %) | |
| Current | 16 (53.3 %) | 28 (50.0 %) | |
| Drinking | | | 0.483 |
| Never | 20 (66.7 %) | 41 (73.2 %) | |
| Former | 1 (3.33 %) | 0 (0.00 %) | |
| Current | 9 (30.0 %) | 15 (26.8 %) | |
| **Sleep parameters** | | | |
| Apnea-hypopnea index, events per h | 42.8 [35.2, 53.4] | 42.1 [36.0, 54.7] | 0.734 |
| Oxygen desaturation index >4 %, per h | 38.2 [31.7, 61.1] | 39.7 [30.4, 56.8] | 0.878 |
| Mean $SaO_2$, % | 92.9 [90.8, 94.1] | 93.0 [91.8, 94.0] | 0.672 |
| Minimum $SaO_2$, % | 84.0 [75.2, 86.0] | 83.0 [77.0, 86.0] | 0.818 |
| Time with $SaO_2$ <90 %, % | 6.75 [1.10, 33.4] | 4.60 [0.90, 12.3] | 0.305 |
| Epworth Sleepiness Scale Score | 5.00 [3.25, 8.00] | 5.00 [4.00, 7.25] | 0.967 |
| **Medical history** | | | |
| Hypertension | 21 (70.0 %) | 30 (53.6 %) | 0.212 |
| Diabetes mellitus | 12 (40.0 %) | 13 (23.2 %) | 0.166 |
| Dyslipidemia | 13 (43.3 %) | 29 (51.8 %) | 0.602 |
| Chronic pneumopathy | 1 (3.33 %) | 3 (5.36 %) | >0.900 |
| Neurological disease | 4 (13.3 %) | 2 (3.57 %) | 0.177 |
| Cerebrovascular disease | 0 (0.00 %) | 0 (0.00 %) | – |
| **Medication** | | | |
| Lipid-lowering drug | 9 (30.0 %) | 18 (32.1 %) | 1.000 |
| Antidiabetics oral medication | 10 (33.3 %) | 11 (19.6 %) | 0.252 |
| Insulin | 3 (10.0 %) | 6 (10.7 %) | >0.900 |
| Antiplatelet and antithrombotic drugs | 8 (26.7 %) | 5 (8.93 %) | 0.054 |
| **Cardiovascular variables** | | | |
| Killip class | | | 0.91 |
| I | 49 (87.5 %) | 26 (86.7 %) | |
| II | 4 (7.14 %) | 3 (10.0 %) | |
| III | 2 (3.57 %) | 1 (3.33 %) | |
| IV | 1 (1.79 %) | 0 (0.00 %) | |
| No. of affected vessels | 1.00 [1.00, 2.00] | 2.00 [1.00, 2.00] | 0.484 |
| No. of stents | 1.00 [1.00, 2.00] | 1.00 [1.00, 2.00] | 0.87 |
| Cardiovascular event type | | | 1.000 |
| Unstable angina | 2 (3.57 %) | 1 (3.33 %) | |
| Non-Q-wave myocardial infarction | 21 (37.5 %) | 12 (40.0 %) | |
| Q-wave myocardial infarction | 33 (58.9 %) | 17 (56.7 %) | |
| Heart rate, bpm | 70.0 [61.0;77.2] | 72.5 [67.0;80.8] | 0.035 |
| Systolic blood pressure, mmHg | 128 [113;140] | 118 [108;128] | 0.095 |
| Diastolic blood pressure, mmHg | 74.0 [67.4;80.0] | 72.8 [64.2;79.9] | 0.768 |
| **Follow-up** | | | |
| Follow-up time, months | 9.11 [3.34, 29.3] | 47.3 [36.2, 64.7] | 0.001* |

Data are n(%) or median [25th percentile, 75th percentile]. *Significant p values ($p < 0.05$). CVE: Cardiovascular event, SaO2: Oxygen saturation, ACS: Acute coronary syndrome.

Table A3) compared to OSA patients who did not suffer a recurrent CVE during follow-up.

For the 38 proteins that were differentially expressed between the groups, we explored the association between protein levels and respiratory polygraphy parameters commonly used for the diagnosis of OSA (AHI, desaturation index, medium and minimum $SaO_2$ and time with $SaO_2$ <90 %). After adjusting for confounding variables (sex, age and BMI), a significant association ($p < 0.05$) of a total of 12 proteins with one or more of the respiratory polygraphy parameters was observed (Fig. A.2B). From the 12 proteins identified, the plasma levels of 11 of them were increased, and one of them was decreased in OSA patients with a recurrent CVE during follow-up (Fig. A.2C and Fig. A.2).

The analysis of the association between differentially expressed proteins and respiratory polygraphy parameters contributed to increasing the degree of evidence of the relationship between OSA and the synthesis of this protein profile.

To increase the degree of evidence of this relationship, we found that the set of the 12 proteins that were differentially expressed in OSA patients with and without recurrent CVE did not discriminate between patients without OSA with and without recurrent CVEs (Table A4). These results contribute to recognizing the specific role of these proteins as biomarkers with discriminatory potential for the recurrence of CVEs in patients with OSA who have developed ACS.(Fig. 2).

### 3.4. Predictive model for recurrent CVEs in patients with ACS and severe OSA

A total of 12 proteins emerged as predictive biomarkers. First, we studied the clustering of the differentially detected proteins that could be separated into three different groups of proteins according to the development of a recurrent CVE during the follow-up (Fig. 3A). From the 12 identified proteins associated with respiratory polygraphy parameters, we performed a random forest to identify the proteins that contribute to the prediction of risk of cardiovascular recurrence (Fig. 3B.1). A multivariate logistic regression model was fitted using the discriminatory set of proteins to predict recurrent CVEs. The 12 proteins were categorized according to their contribution to maximizing the discrimination ability for the prediction of cardiovascular recurrence. Finally, the analysis of the 3 proteins with the highest contribution for prediction provided a discriminatory predictive model for such recurrent CVEs (AUC (95 % CI), 0.81 (0.71–0.9)) (Fig. 3B.2).

### 3.5. Pathway enrichment analysis

For in silico analysis, we explored the set of 12 proteins associated with respiratory polygraphy parameters that discriminated between OSA patients with and without recurrent CVEs. To interpret the biological function of the identified proteins, protein–protein relationships and enrichment pathway analysis were retrieved from STRING. Pathway enrichment analysis revealed 43 pathways to be enriched for OSA patients with recurrent CVE (Table A5). The 25 top significantly enriched pathways are depicted in Fig. 4A with an FDR cutoff of 0.05. Additionally, to analyze their biological function, functional enrichment analysis was performed with Reactome software. Selected pathways significantly enriched in OSA patients with a recurrent CVE (in comparison to OSA patients without recurrent CVE) are depicted in Table A6. A total of 53 significant pathways were found with an FDR cutoff of 0.05. Proteins were implicated in pathways associated with proliferation, communication and apoptosis cell processes, along with regulation/response to external and internal stimulus, cellular protein metabolic process, inflammation, and immune system. As a complement to STRING and the Reactome, the study of the location in the different tissues/organs was performed by using GTEx. The identified proteins showed generalized expression in the lung and heart and in other tissues and organs (Fig. 4B).

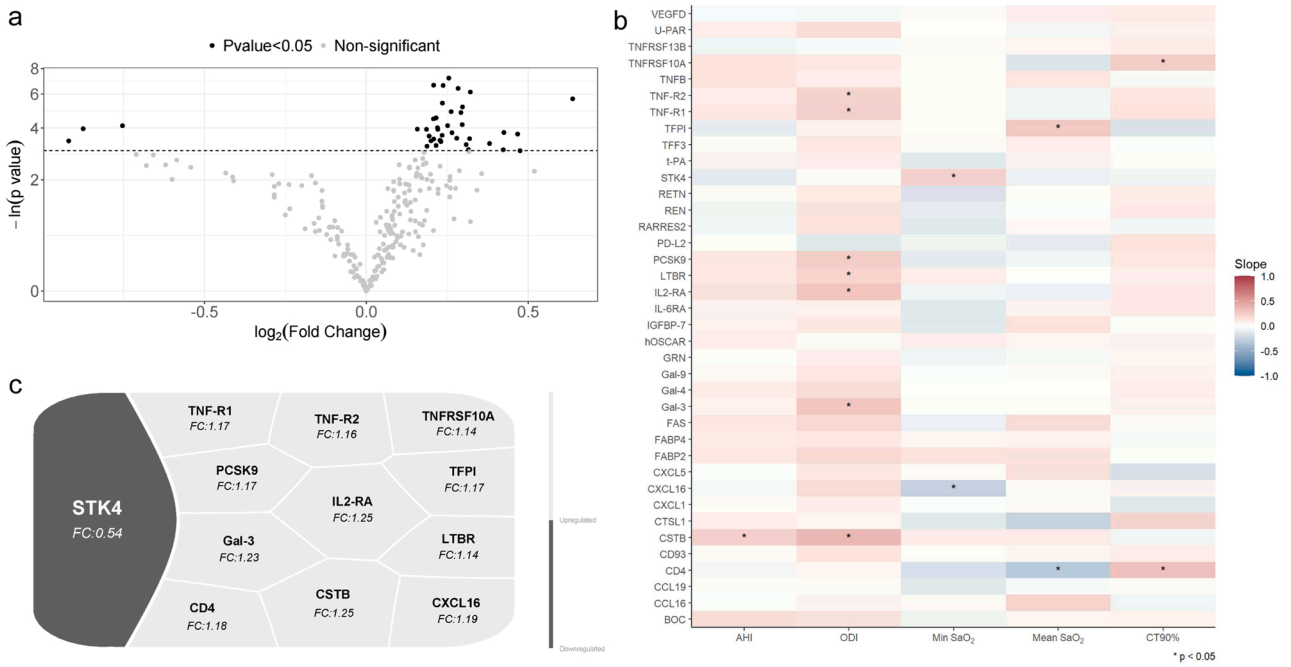


**Fig. 2.** Differential protein plasma levels according to the risk of recurrent CVE in severe OSA patients. (a) Volcano plot shows the negative logarithm of the p value versus the $\log_2$ fold change for each detected protein. The horizontal dashed line indicates a cutoff of 0.05 for the p value. Black dots indicate proteins with significant differences in plasma levels between the groups. (b) Linear association between identified proteins and respiratory polygraphy parameters adjusted by confounding factors (age, sex and BMI). The slope between the identified proteins and respiratory polygraphy parameters is represented by the color scale, with red tones related to positive associations and blue tones related to negative associations. *Significant p-values ($p < 0.05$). (c) Voronoi treemaps of differentially expressed proteins. The sizes of the polygons reflect the magnitude of the fold change. Upregulated or downregulated proteins grouped by identical color schemes are shown in the figure, along with the name of each protein. OSA: Obstructive sleep apnea, AHI: Apnea-hypopnea index, ODI: Oxygen desaturation index, $SaO_2$: Oxygen saturation, CT90 %: Time with $SaO_2$ below 90 %.

## 4. Discussion

In this post-hoc analysis of the ISAACC study, we identified specific inflammatory and cardiovascular disease protein biomarkers associated with severe OSA in patients with a first ACS and recurrent CVE. The results of this study indicate that, in patients with ACS and recurrent CVE, the presence of severe OSA influences the proteomic profile associated with CVD, suggesting that OSA would exert a deleterious role in specific endotypes of patients who have suffered an ACS. The proteins identified were associated with respiratory polygraphy parameters commonly used for the diagnosis of OSA and were implicated in biological pathways such as cell proliferation, communication, apoptosis, and regulation of inflammation and the immune system with a potential role in destabilizing atherosclerotic plaques. Finally, our results showed a proteomic profile that could predict a high risk of having a recurrent CVE in patients with severe OSA.

This study delves into the molecular characterization of the effect of OSA on the promotion of biomarkers of cardiovascular risk in endotypes in which OSA exerts a deleterious effect. Our findings revealed a set of 38 proteins measured in plasma that were differentially expressed in OSA patients with a recurrent CVE compared with OSA patients without a recurrent CVE. In addition, a significant association was found between the 12 proteins that were differentially expressed in OSA patients and respiratory polygraphy parameters. Furthermore, we confirmed that this protein profile was not dysregulated in ACS patients without OSA, suggesting that this specific protein profile would be influenced by OSA.

Recent randomized controlled trials have failed to demonstrate a positive effect of treatment with CPAP on secondary cardiovascular prevention [13,22,23]. Moreover, in the ISAACC study that also included patients without OSA, it was reported that OSA does not increase the risk of the incidence of CVEs in the population with ACS. However, a later post-hoc analysis from the ISAACC trial reported a deleterious effect of OSA in terms of the risk of recurrent CVEs in patients admitted for a first ACS without previous CVD [12]. It has been largely hypothesized that the deleterious effect of OSA is not homogeneous in all patients, with some patients being more susceptible to the deleterious effects of OSA [24]. The present study aims at the potential deleterious effect of OSA in specific endotypes of patients with an increased risk of recurrent CVE. Since the baseline clinical data of the study cohort did not allow us to predict or know the risk of cardiovascular recurrence in each patient, even the severity of the first ACS was not different between groups. The identification of altered levels of protein biomarkers associated with CVD and inflammation in patients with recurrent CVEs and OSA would contribute to identifying the potential promoting role of OSA in the recurrence of CVEs. In addition, the identification of a specific biomarker profile in patients at risk of recurrence of CVEs would contribute to improving the clinical management of the patient and would indicate the need to know the potential beneficial effect of CPAP treatment for OSA in those at risk of a recurrent CVE in specific endotypes. Moreover, future studies are needed to explore specific proteomic profiles related to OSA in others endotypes such as patients with previous CVD.

OSA promotes the manifestation of CVD [2]. Clinical and experimental evidence suggests that OSA consequences promote CVDs through specific intermediate mechanisms, such as inflammation, endothelial dysfunction, hypercoagulability, oxidative stress and metabolic dysfunction [25,26]. From animal model studies it has been also postulated that exposure to intermittent hypoxia promotes cardiovascular remodeling that is reversed after normoxia restoration [27]. This finding suggests that remodeling induced by severe chronic intermittent hypoxia is affected by the age at which chronic intermittent hypoxia onset occurs, suggesting that the deleterious cardiovascular effects associated with chronic intermittent hypoxia may be more pronounced in younger populations [27]. Although the mechanisms by which OSA

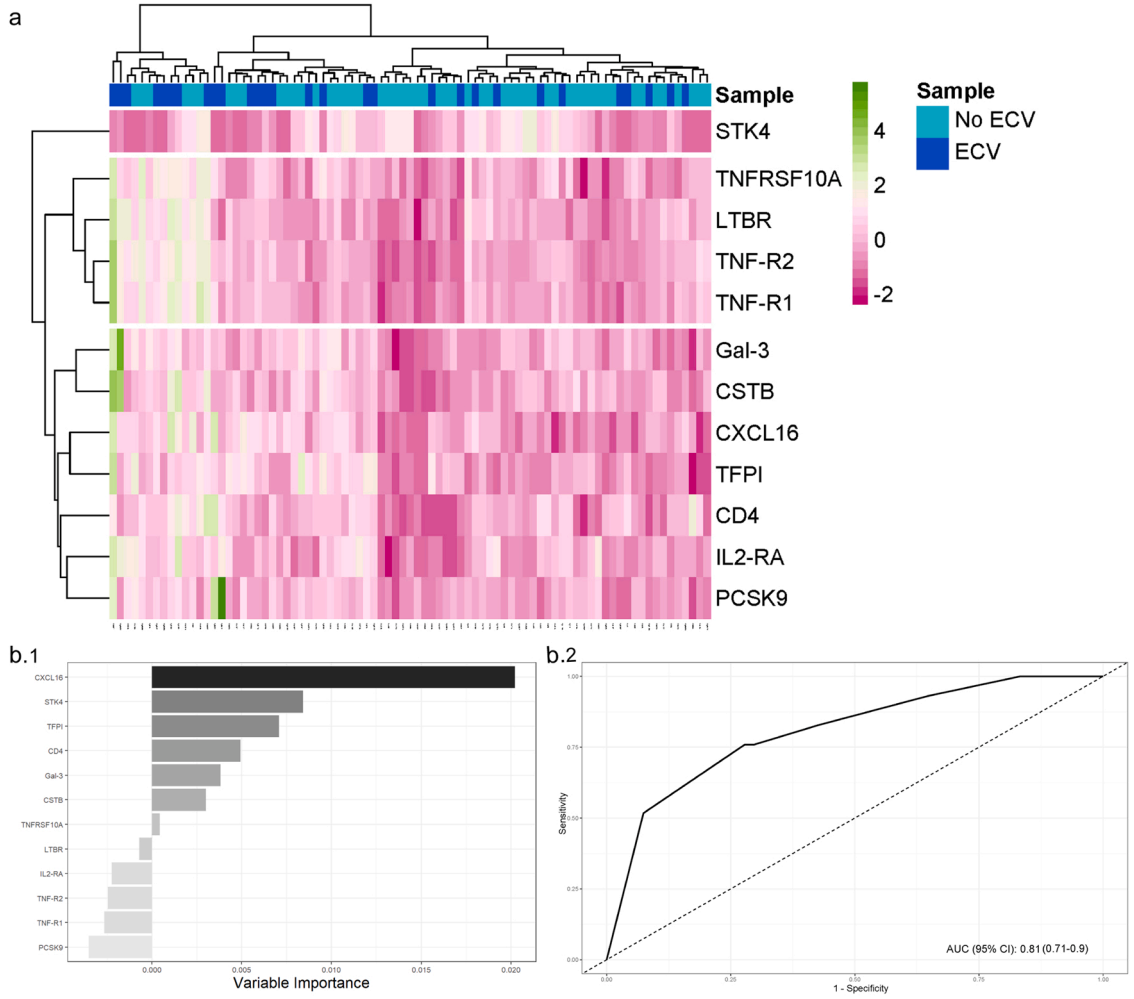


**Fig. 3.** Plasma proteomic profile associated with recurrent cardiovascular risk in severe OSA patients. (a) Hierarchical clustering heatmap. Each column refers to an OSA patient sample and each row represents the identified proteins. The patient clustering tree is plotted on top. The protein clustering is shown on the left. Protein levels are represented through a color scale, with green/pink representing up/down-regulation. (b.1) Proteins are ranked by their variable importance. (b.2) ROC curve for the protein profile (CXCL16, STK4 and TFPI). The discriminative power of the profile was assessed using the area under the curve (AUC) and 95 % confidence interval. CVE: Cardiovascular event, OSA: Obstructive sleep apnea.

promotes cardiovascular disease have been extensively studied and described, few studies have explored whether the manifestation of these mechanisms occurs homogeneously in all patients with OSA. Different factors have been postulated that could influence the deleterious effect of OSA. Thus, different studies indicate that younger patients would be more vulnerable to the deleterious effects of OSA and would present the highest risk of mortality [28]. From observational studies, it has been reported that older subjects might be protected against the harmful effects of OSA. Thus, studies such as the Sleep Heart Health Study have shown that the AHI was associated with hypertension, diabetes, dyslipidemia, and cardiovascular disease only in patients under 65 years of age [29]. In addition, from the ISAACC post-hoc analysis [12] and others [30], it can be postulated that a remarkable comorbid condition might exert a ceiling effect on the extent to which OSA could increase the risk of recurrent CVEs. In contrast, the deleterious cardiovascular effects associated with OSA would manifest in those patients with a low comorbid load. It is remarkable that concomitant CVD and the OSA synergistic effect revealed in available studies [31–33] would not be homogeneous in all OSA patients. In fact, a recent study reported a sex divergence in the risk CVE recurrence associated to OSA and only female patients showed an incremental risk of CVE recurrence [32]. In our study, regardless a lack of statistical significance, we found a higher percentage of females with recurrent CVE. All the above might indicate the importance of exploring and identifying the high-risk endotype that would be vulnerable to the deleterious effect of OSA and that might be determined, among other causes, by the impact of the comorbidity burden of the patient.

The identified proteomic profile in the present study is involved in biological pathways that promote cell migration, regeneration and apoptosis, oxidative stress and the inflammatory response, among others. OSA was found to be related to oxidative stress, by increasing the synthesis of reactive oxygen species [8,34,35]. At the same time, this increase entails the development of atherosclerosis, which stimulates vascular endothelial cell structural damage and dysfunction [36]. In addition, OSA was also related to inflammation in different aspects [35, 37,38]. Consequently, the proteins identified are related to inflammatory pathways. The role of tumor necrosis factor in sleep regulation has been observed through the activation of the NF-κB pathway [39–41]. Other related inflammatory pathways regulated by our set of proteins included several interleukins, such as IL-2, IL-3, IL-8 and IL-10 [40–43]. These findings corroborate the previously reported implication of OSA in systemic inflammation, driving endothelial activation and dysfunction and ultimately leading to cardiovascular consequences. Understanding the molecular mechanisms of potential circulating plasma biomarkers in OSA patients could contribute to the acknowledgment of OSA mechanisms that trigger recurrent CVEs.

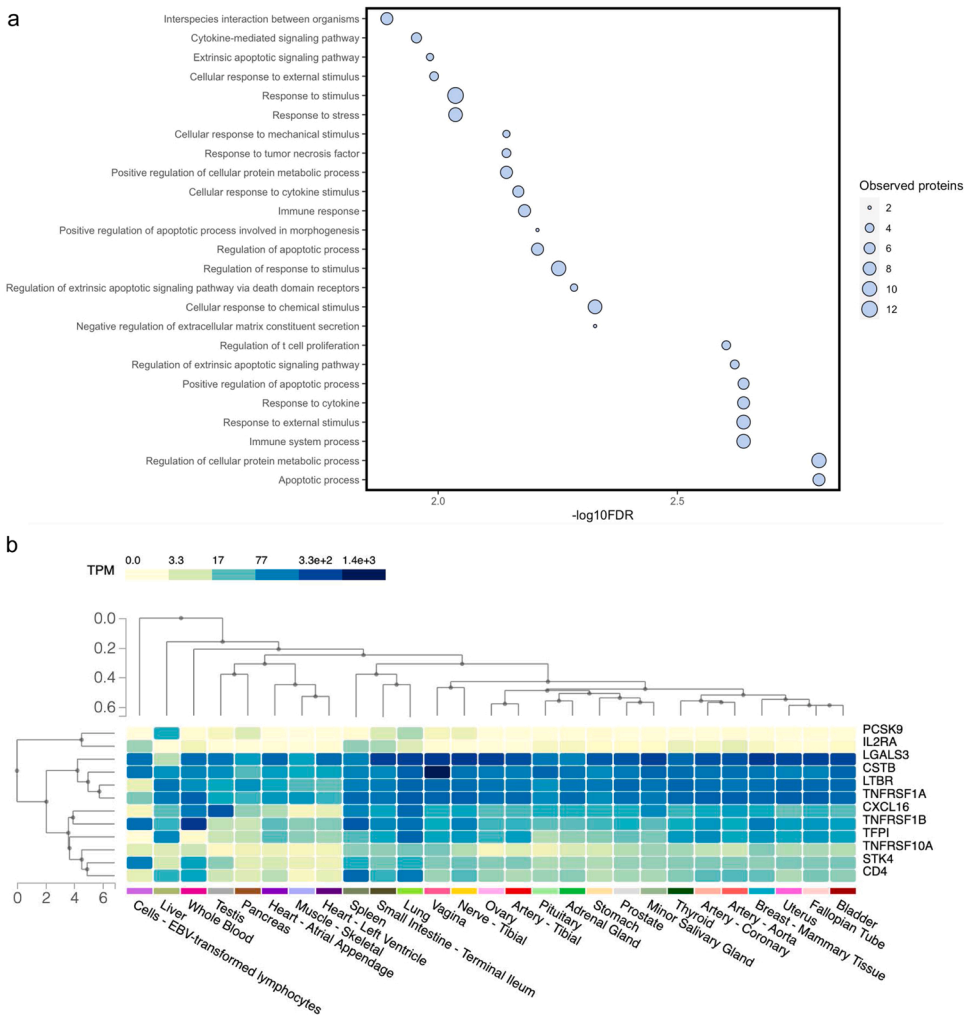


**Fig. 4.** Gene set enrichment analysis and cellular/tissue and organ expression. (a) Gene set enrichment analysis considering proteomic signatures associated with recurrent CVE using STRING. The graph shows the false discovery rate values of the top twenty-five GO terms. The size of each point is proportional to the number of identified proteins and is associated with sleep parameters that participate in the biological process. (b) Tissue and organ-enrichment analysis using genotype-tissue expression (GTEx). The hierarchical clustering shows tissues and/or organs on the bottom and proteins on the right.

Our results also suggest that the proteomic fingerprint would contribute to the prediction of the risk of cardiovascular recurrence in patients with ACS and severe OSA (AUC (95 %CI): 0.81 (0.71–0.9)). In particular, this potential predictive signature is composed of three proteins: CXCL16, STK4 and TFPI. These three proteins are widely described as cardiovascular risk factors. CXCL16 plays an important role in atherosclerosis and plaque destabilization [36,44,45]. STK4 is a key component of the Hippo signaling pathway and is thus an essential regulator of cardiac development and regeneration/repair after injury [46,47]. TFPI is implicated in the development of atherosclerosis [48,49] and is associated with fibrinogen levels [50,51]. In addition, a previous study of OSA patients compared to non-OSA patients reported a profile of 65 proteins, highlighting a differentially expressed profile pattern of TFPI in patients with OSA [52]. These pathways have been previously described as targets of the pathophysiological consequences of OSA [37,40,50,53]. Specifically, these three proteins play a key role in regulating the inflammatory response and oxidative stress, together with an effect on endothelial dysfunction. Cycles of oxygenation and reoxygenation of hypoxemia generate a high production of free reactive oxygen species, inducing oxidative stress, increasing systemic inflammation and sympathetic activity, and enhancing possible adverse cardiovascular consequences [54]. These free reactive oxygen species modulate a wide variety of transcription factors and signaling pathways that promote the expression of adhesion and inflammatory molecules, promoting endothelial dysfunction and exacerbating atherosclerosis [55]. Considering all of above, the role of these biomarkers might be related to the consequences and implications of OSA.

The strengths of the study comprise its multicentric design that includes a prospective cohort with a relatively long follow-up time. Moreover, we used a targeted PEA technology, which showed a high reproducibility of the measurements performed in the present study (Fig. A1). In addition, the identification of a molecular profile of proteins whose expression is correlated with polygraphy variables commonly used for the diagnosis of OSA, reinforces the potential promoting role of OSA in the expression of these biomarkers related to the risk of CV recurrence in patients with ACS. The study presents some limitations that deserve comment. First, the observational nature of the present study prevents determining conclusions about the causative role of OSA in promoting cardiovascular damage in certain patient endotypes of patients with ACS. Nevertheless, the observed association of reported cardiovascular risk biomarkers with OSA-specific variables contributes to reinforcing the potential role of OSA as a promoter of the development of recurrent events in certain patients with severe OSA and ACS without a previous history of cardiovascular disease. Moreover, we found that the set of proteins that were differentially expressed in OSA patients with and without recurrent CVEs, did not discriminate patients without OSA with and without recurrent CVEs. Second, the results of this study indicate the possibility of identifying biomarker profiles associated with different risks of recurrent CVEs in patients who have suffered a first ACS and without previous CVD. Moreover, the relatively small sample size of the group of patients could induce an underlying bias in our study that limit the representativeness of this population. These exploratory results should be considered with caution and need to be validated in independent cohorts. Third, although in the present

study we described the evaluation of the proteomic profile associated with the risk of recurrent CVE in patients with severe OSA future studies should explore the role of proteomics in predicting CVEs in ACS patients with mild or moderate OSA. Fourth, some biomarkers appear to have prognostic importance; nevertheless, their implementation in clinical practice may be limited by the differences in design and size of the different studies. Fifth, the results from the present study may not be extrapolated to a different population from that of patients admitted to the hospital for ACS. This fact makes it necessary to specifically identify endotypes in patients with OSA who are seen in other clinical settings.

## 5. Conclusions

In patients with a first ACS and severe OSA, a specific proteomic profile was associated with recurrent CVEs. This proteomic profile was correlated with respiratory polygraphy parameters commonly used for the diagnosis of OSA. The proteins were implicated in biological pathways, including cell proliferation, communication and apoptosis and regulation/response to the inflammatory and immune systems. Finally, proteomic profiling of patients with OSA and ACS would constitute a strategy to predict recurrent CVEs that would potentially contribute to the patient risk stratification and clinical management.

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## CRediT authorship contribution statement

AZ, ES, FB and MS contributed to the conceptualization and methodology, GT, OMi, LP, AC, DM, JA, JD, AU, OMe, MJM, EO, JM, MP, MM, RC, JMM, EC and FB contributed to the resources and data curation; AZ, ES, IB and MS contributed to formal analysis, investigation, interpretation and validation; and all authors contributed to the writing-Original Draft of the manuscript and critically revised the manuscript for important intellectual content and approved the final version. MS is the guarantor of the paper.

## Conflict of interest statement

FB received a research grant from ResMed (an Australian company that develops products related to sleep apnea), the Health Research Fund, the Spanish Ministry of Health, the Spanish Respiratory Society, the Catalonian Cardiology Society, Esteve-Teijin (Spain), Oxigen Salud (Spain), and ALLER to develop the ISAACC trial. ResMed partly funded the ISAACC study but did not participate in nor was involved in decisions regarding study development or the present manuscript. All other authors declare no competing interests.

## Data Availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.114125.

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