Received: 25 June 2021 | Revised: 26 August 2021 | Accepted: 21 September 2021

DOI: 10.1111/eci.13685

ORIGINAL ARTICLE

WILEY

Adipose tissue and blood leukocytes ACE2 DNA methylation in obesity and after weight loss

Andrea G. Izquierdo[1,2,3] | **Marcos C. Carreira**[2,3,4] | **Hatim Boughanem**[3,5] | **Jose M. Moreno-Navarrete**[3,6] | **Carolina F. Nicoletti**[7] | **Paula Oliver**[3,8] | **Daniel de Luis**[9] | **Carla B. Nonino**[7] | **Maria P. Portillo**[3,10] | **Miguel A. Martinez-Olmos**[1,2,3] | **Jose M. Fernandez-Real**[3,6] | **Francisco J. Tinahones**[3,5] | **J. Alfredo Martinez**[3,11,12] | **Manuel Macias-González**[3,5] | **Felipe F. Casanueva**[2,3,4] | **Ana B. Crujeiras**[1,2,3]

[1]Epigenomics in Endocrinology and Nutrition Group, Epigenomics Unit, Instituto de Investigacion Sanitaria de Santiago de Compostela (IDIS), Complejo Hospitalario Universitario de Santiago de Compostela (CHUS/SERGAS), Santiago de Compostela, Spain

[2]Endocrine Division, Complejo Hospitalario Universitario de Santiago de Compostela (CHUS/SERGAS) and Santiago de Compostela University (USC), Santiago de Compostela, Spain

[3]CIBER Fisiopatologia de la Obesidad y Nutricion (CIBERobn), Madrid, Spain

[4]Molecular Endocrinology Group, Instituto de Investigacion Sanitaria de Santiago de Compostela (IDIS), Complejo Hospitalario Universitario de Santiago de Compostela (CHUS/SERGAS) and Santiago de Compostela University (USC), Santiago de Compostela, Spain

[5]Department of Endocrinology and Nutrition, Virgen de la Victoria University Hospital, University of Malaga (IBIMA), Malaga, Spain

[6]Department of Diabetes, Endocrinology and Nutrition, Institut d'Investigació Biomèdica de Girona (IdIBGi) and Universitat de Girona, Girona, Spain

[7]Department of Internal Medicine, Laboratory of Nutrigenomic Studies, Ribeirao Preto Medical School (FMRP) University of Sao Paulo (USP), Sao Paulo, Brazil

[8]Nutrigenomics and Obesity Group, University of the Balearic Islands and Health Research Institute of the Balearic Islands (IdISBa), Palma, Spain

[9]Center of Investigation of Endocrinology and Nutrition, Medicine School and Department of Endocrinology and Investigation, Hospital Clinico Universitario, University of Valladolid, Valladolid, Spain

[10]Nutrition and Obesity Group, Department of Nutrition and Food Science, Lucio Lascaray Research Institute and Bioaraba Health Research Institute, University of the Basque Country (UPV/EHU), Vitoria, Spain

[11]Department of Nutrition, Food Science and Physiology, Centre for Nutrition Research, Navarra Institute for Health Research, University of Navarra (UNAV) and IdiSNA, Pamplona, Spain

[12]Nutritional Genomics and Epigenomics Group, IMDEA Food, CEI UAM + CSIC, Madrid, Spain

Correspondence
Ana B. Crujeiras, Instituto de Investigación Sanitaria, Complejo Hospitalario de Santiago (CHUS). C/ Choupana, s/n. 15706. Santiago de Compostela, Spain.
Email: anabelencrujeiras@hotmail.com

Funding information
PronoKal Group; Agencia de Innovación y Desarrollo de Andalucía, Grant/Award Number: RC-0001-2018 and C-0029-2014; Instituto de Salud Carlos III, Grant/

Abstract

Background: Obesity was consistently associated with a poor prognosis in patients with COVID-19. Epigenetic mechanisms were proposed as the link between obesity and comorbidities risk.

Aim: To evaluate the methylation levels of angiotensin-converting enzyme 2 (ACE2) gene, the main entry receptor of SARS-CoV-2, in different depots of adipose tissue (AT) and leukocytes (PBMCs) in obesity and after weight loss therapy based on a very-low-calorie ketogenic diet (VLCKD), a balanced hypocaloric diet (HCD) or bariatric surgery (BS).

Award Number: PI17/01287, PI20/00650, PI20/00628 and CP17/00088; Consellería de Cultura, Educación e Ordenación Universitaria, Xunta de Galicia, Grant/ Award Number: IN607B2020/09

Materials and Methods: DNA methylation levels of ACE2 were extracted from our data sets generated by the hybridization of subcutaneous (SAT) ($n = 32$) or visceral (VAT; $n = 32$) adipose tissue, and PBMCs ($n = 34$) samples in Infinium HumanMethylation450 BeadChips. Data were compared based on the degree of obesity and after 4–6 months of weight loss either by following a nutritional or surgical treatment and correlated with ACE2 transcript levels.

Results: As compared with normal weight, VAT from patients with obesity showed higher ACE2 methylation levels. These differences were mirrored in PBMCs but not in SAT. The observed obesity-associated methylation of ACE2 was reversed after VLCKD and HCD but not after BS. Among the studied CpG sites, cg16734967 and cg21598868, located at the promoter, were the most affected and correlated with BMI. The observed DNA methylation pattern was inversely correlated with *ACE2* expression.

Conclusion: Obesity-related VAT shows hypermethylation and downregulation of the *ACE2* gene that is mirrored in PBMCs and is restored after nutritional weight reduction therapy. The results warrant the necessity to further evaluate its implication for COVID-19 pathogenesis.

KEYWORDS

bariatric surgery, COVID-19, epigenetics, obesity, VLCKD

1 | INTRODUCTION

In the era of the pandemic COVID-19 disease, excess body weight was significantly associated with severe forms of the disease, independent of its classical associated comorbidities.[1,2] It was observed and reported by international groups that overweight, obesity and morbid obesity were directly associated with higher infection rates, worse evolution at hospital and increased probability of death.[3,4] However, it is not yet known why excess adiposity is associated with worse outcome following coronavirus infection.[5] Knowledge of the mechanistic connection between excess body weight and the risk of developing severe forms of COVID-19 will enable the identification of patients at higher risk and provide tools for protecting this most disadvantaged group of patients.[1]

The most critical features for viral transmission and potential therapeutic targets are the cellular factors used for virus entry. It was discovered that SARS-CoV-2 uses ACE2 receptor for entry into cells during the process of infection.[6] Since this discovery, scientific efforts have focussed on the function of ACE2 on the risk and development of COVID-19.[7] On the other hand, since its discovery 20 years ago, ACE2 was also associated with a protective role for counteracting several pathological processes such as cardiorenal[8] or liver disorders.[9] Moreover, ACE2 exerts beneficial effect on glycolipid metabolism and thermogenesis.[10]

Adipose tissue (AT) has been proposed as a relevant player among the potential mechanisms involved in the association between obesity and COVID-19 severity.[7] Particularly, visceral adipose tissue (VAT) plays a leading role in the context of obesity comorbidities.[11] Weight loss treatments considerably reduce these intrinsic pathological conditions within AT and, therefore, also decrease the health risks.[12-14] Unravelling the molecular mechanisms underlying the role of AT could provide tools for the prevention and management of COVID-19 in the most susceptible group of patients in the form of more personalized and precise treatment.

Epigenetic research has revealed that global DNA methylation along with the *ACE2* gene sequence, noncoding RNA and post-translational histone modifications, may drive differences in individual responses to viral infection.[15-20] In light of these findings and the association between epigenetic mechanisms and obesity comorbidities risk, we hypothesized that the contribution of AT to COVID-19 severity could be mediated by epigenetic mechanisms.

The search for epigenetic biomarkers for the diagnosis and management of diseases presents a challenge in that the target tissue is usually inaccessible without surgery, such as in the case of AT in obesity research. In lieu of AT, epigenetic markers might be detectable in easily accessible samples, such as peripheral blood cells,[21] which are increasingly being used in clinical and obesity research to identify risk biomarkers.[22-26]

This study aimed to (1) evaluate and compare methylation levels in *ACE2* in SAT and VAT depending on obesity status, (2) validate the observations in PBMCs and (3) determine the effect of weight loss therapy based on very-low-calorie ketogenic diet (VLCKD), a hypocaloric diet or bariatric surgery on the obesity-related methylation levels of *ACE2*.

2 | MATERIALS AND METHODS

2.1 | Data collection and DNA methylation assessment

The DNA methylation profile of *ACE2* was evaluated by analysing our different methylome data sets of SAT, VAT and PBMCs as published[21,27-29] plus unpublished data, as described in the Figure S1. The methylome data were obtained through the hybridization of samples in the Illumina Infinium HumanMethylation450 BeadChip or the Illumina Infinium MethylationEPIC BeadChip (Illumina) depending on the cohort. These data were overlapped and commonly detected CpGs with a valid signal ($p < .01$) in all samples included in this study were selected to evaluate the differential methylation levels based on obesity phenotypes, obesity-related comorbidities and after weight loss therapies. Obesity phenotypes were defined based on individual BMI as normal weight (BMI < 25), obesity (BMI ≥ 25- < 35) and severe obesity (BMI ≥ 35). The characteristics of patients included in this study are shown in Table S1.

Changes in methylation levels after weight loss therapies were explored using data sets of blood leukocyte-derived DNA from patients with obesity underwent a VLCKD (PnK method®),[30] a short-term hypocaloric diet[28] or bariatric surgery.[29] Methylation data were extracted from DNA samples of these patients before and 6 months after weight loss therapies that were hybridized to the Illumina Infinium MethylationEPIC BeadChip.

The study protocols from which the data were collected were performed in accordance with the Declaration of Helsinki and were approved by the corresponding Ethics Committee for Clinical Research: Clinica Universidad de Navarra Ethics Committee (CEI-UN), Ethics Committee of the University Hospital of Girona (Spain), Ethics Committee of Clinical Research of Galicia (Spain), Ethics Committee of the University Hospital of Malaga (Spain) and Ethics Committee of the University Hospital of Valladolid (Spain). Written consent has been obtained from each patient or subject after full explanation of the purpose and nature of all procedures used.

For all samples, DNA extraction, bisulfite conversion and the hybridization, as well as data normalization, quality filtering and statistical calculations were carried out following the protocols according to the manufacturer's instructions as previously described.[31,32] Reporting of the study conforms to broad EQUATOR guidelines.[33]

2.2 | Data collection and gene expression assessment

AT *ACE2* gene expression data were obtained from a previously published gene expression microarray data set deposited in the Gene Expression Omnibus database (GSE15524 GEO COHORT). Briefly, this study consisted in individualized analysis through expression profiling of 20,000 probes in 28 tissue samples obtained during surgical intervention and evaluated in SAT and VAT. Patient samples from men and women of varying body size (normal weight to morbid obesity) were collected at the time of operation in the fasting state.

The expression of *ACE2* in PBMCs samples from transversal and a longitudinal cohorts of patients was measured as published.[34] Differential expression of the *ACE2* gene was quantified based on the obesity status with respect to normal weight. In addition, expression data were quantified in obese patients undergoing weight loss intervention using a VLCKD (PnK method®). In both cohorts, gene expression was quantified using TaqMan real-time PCR as previously described.[14,35,36] Gene expression levels were normalized to *GAPDH* as an internal control and expressed as the average value for the control group based on the $2^{-\Delta\Delta Ct}$ method. The real-time qPCR experiments were performed in accordance with the Minimum Information Guidelines for the Publication of Quantitative Real-Time PCR Experiments (http://www.rdml.org/miqe). The following commercially available and pre-validated TaqMan primer/probe sets were used: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs02758991_g1, Applied Biosystems) and angiotensin-converting enzyme 2 (*ACE2*, Hs01085333_m1; Applied Biosystems).

Further analysis in *ACE2* transcript levels after following a hypocaloric diet or bariatric surgery was obtained and analysed from previously published gene expression microarray data sets deposited at the Gene expression omnibus (E-GEOD-18897 cohort and EGEOD-32575 cohort respectively).

2.3 | Statistical analysis

The sample size of the current study was calculated to detect differences for methylation levels taking into account published values of epigenome-wide analysis in the field of obesity.[21,37,38] It was calculated for an effect of size ≥2%

in methylation levels, and $\alpha = .05$, and a power $(1-\beta)$ of 80%. The sample size provided sufficient power to test for effects on methylation levels and other metabolic variables of interest.

The selected CpG sites of *ACE2* gene were previously filtered using the Genome Studio Illumina software (V2010.3). The global methylation level in the *ACE2* gene, measured at the CpG sites present in all data sets, was compared between the different study groups which were classified based on BMI using univariate ANOVA and a Bonferroni post hoc analysis. Differential *ACE2* methylation levels following a VLCKD, a hypocaloric diet or bariatric surgery, were measured using repeated measures ANOVA. The potential association between the BMI and DNA methylation levels of cg16734967 and cg21598868 was evaluated using the Pearson coefficient test. Differences in *ACE2* gene expression levels between the groups comprising subjects with normal weight, obesity and morbid obesity, as well as the group of patients with obesity subjected to VLCKD, hypocaloric diet or bariatric surgery were evaluated using univariate ANOVA and repeated measures ANOVA respectively. No association was found between global methylation levels of ACE2 gene in SAT, VAT nor PBMCs and age ($p = .112$, $p = .702$, $p = .056$ respectively) or sex ($p = .161$, $p = .822$, $p = .206$ respectively). All data are expressed as mean ± standard deviation (SD) in the tables and as mean ± standard error of the mean (SEM) in the figures. $p < .05$ was considered statistically significant, and a value of $p \leq .1$ was considered indicative of a trend of significance. Statistical analyses were performed using SPSS 25.0 software (SPSS Inc) for Windows 10 (Microsoft) and GraphPad Prism 7.0 software (GraphPad Software Inc).

3 | RESULTS

3.1 | Comparison of *ACE2* methylation levels and gene expression in AT according to obesity phenotypes

The methylation levels in CpG sites in the promoter, body and 3′UTR of the *ACE2* gene in human AT were evaluated and compared based on obesity status (Figure 1A). No statistically significant differences in *ACE2* methylation levels were observed in SAT in the different obesity phenotypes, except for the CpGs located at body that showed higher methylation levels in obesity and morbid obesity group than in normal weight group (Figure 1B). In contrast, *ACE2* methylation level in VAT was statistically higher in the obesity and morbid obesity group than in the normal weight group in most of studied CpG sites in the promoter and 3′UTR regions, while in the body, methylation levels were lower in morbid obesity group than in normal weight group (Figure 1C).

Because DNA methylation affects transcription, *ACE2* mRNA level was examined based on the microarray analysis reported in SAT and VAT.[39] Relevantly, *ACE2* expression was lower in the obesity group than in normal weight group, with statistical significance particularly in VAT (fold change of −.39; $p = .002$), while in SAT the expression of *ACE2* was not affected (fold change of −.09, $p = .693$).

3.2 | Comparison of *ACE2* methylation levels and gene expression in PBMCs according to obesity phenotypes and in response to weight loss intervention

The analysis of the *ACE2* methylation levels in PBMCs mirrored the results observed in VAT (Figures 1C and 2A). It was detected particularly at CpG sites located at the promoter (cg05748796, cg08559914, cg16734967, cg18877734, cg21598868) and 3′UTR regions (cg23232263). When ACE2 gene expression was evaluated in PBMCs, also a downregulation was observed in the obesity groups (Figure 2E).

Relevantly, the analysis of the *ACE2* methylation levels in PBMCs of patients with obesity undergoing a VLCKD revealed a decrease at the maximum ketosis point (mean of 9 kg of body weight loss) and endpoint phase (mean of 20 kg of body weight loss) compared with basal (Figure 2B). Differences were statistically significant in two CpGs located at the promoter of the gene (cg16734967, $p = .015$ at Maximum Ketosis and $p = .021$ at Endpoint; cg21598868, $p = .022$ at Maximum Ketosis and $p = .003$ at Endpoint compared with basal levels). Statistically significant differences were also observed in ACE2 methylation levels after following a hypocaloric diet[28] in a CpGs (cg08559914, $p = .019$) and a trend in two CpGs (cg18877734, $p = .083$; cg21598868, $p = .095$; Figure 2C). By contrast, in patients with obesity underwent bariatric surgery,[29] no statistically significant differences were observed in *ACE2* methylation levels, despite a reduction of 23 kg in body weight (Figure 2D).

These weight loss-induced decreases in methylation were correlated with an increase in *ACE2* mRNA expression after the VLCKD (Figure 2F) and after the short-term hypocaloric diet (Figure 2G) compared with basal levels. These differences reached statistical significance at 6 weeks ($p = .010$) and a trend towards statistical significance at 3 weeks ($p = .059$) after a hypocaloric diet and at 4 weeks after a VLCKD ($p = .096$), whereas similar transcript levels than baseline were observed after bariatric surgery ($p = .419$; Figure 2H).

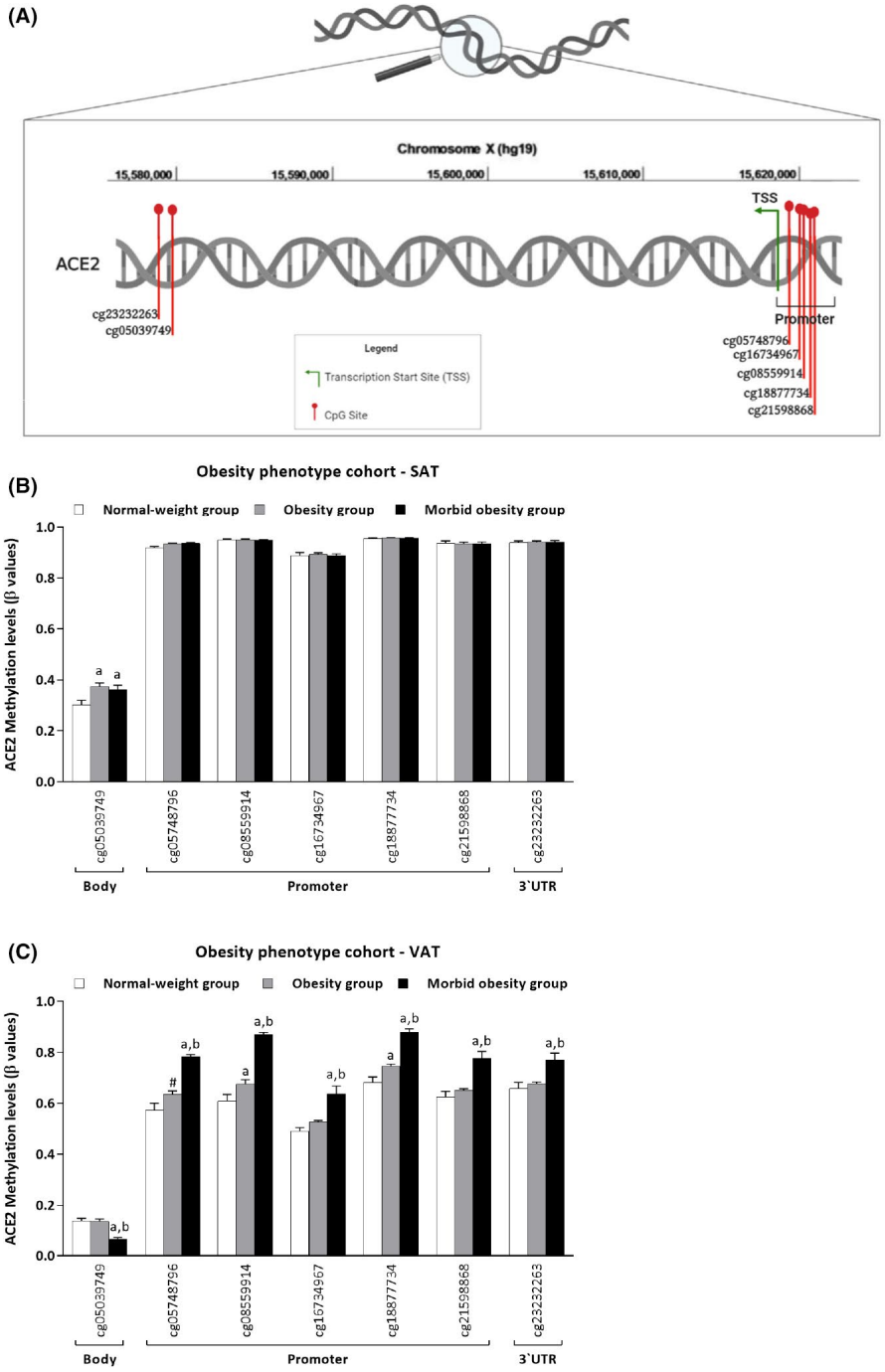

FIGURE 1 Analysis of *ACE2* methylation levels of gene in adipose tissue (AT). (A) Map of a DNA fragment from *ACE2* gene sequence with nucleotides upstream (−) and downstream (+) of the transcription start site (TSS) containing the examined CpG sites represented by the target IDs of the Infinium Human Methylation 450 BeadChip array and located at the body, promoter and 3′UTR of the gene sequence. (B) *ACE2* methylation levels based on obesity phenotypes in subcutaneous adipose tissue (SAT). (C) *ACE2* methylation levels based on obesity phenotypes in visceral adipose tissue (VAT). Statistically significantly differences between groups were analysed using ANOVA and a Bonferroni post hoc analysis. Data show mean ± SEM (standard error of the mean) from normal weight group ($n = 8$ SAT; $n = 9$ VAT), obesity group ($n = 13$ SAT; $n = 11$ VAT) and morbid obesity group ($n = 11$ SAT; $n = 12$ VAT). [a]denotes statistically significant differences when compared with those in the normal weight group, [b]denotes statistically significant differences when compared with those in the obesity group. [#]denotes a trend towards statistical significance when compared with those in the normal weight group

3.3 | Differential *ACE2* methylation levels based on obesity-related insulin resistance and nonalcoholic steatohepatitis

Considering that obesity comorbidities could also induce changes in the epigenetic regulation of *ACE2*, its methylation profile was evaluated based on the insulin resistance status in VAT[27] or on the nonalcoholic steatohepatitis (NASH) status in PBMCs. Again, in these cohorts, statistically higher methylation levels were observed in the obesity groups compared with the normal weight group, independently of insulin resistance (Figure 3A) or NASH (Figure 3B) status.

3.4 | Association between *ACE2* methylation levels and BMI in VAT and PBMCs

Taking all data together, two CpG sites located at ACE2 promoter region (cg16734967 and cg21598868) were commonly statistically significantly different based on

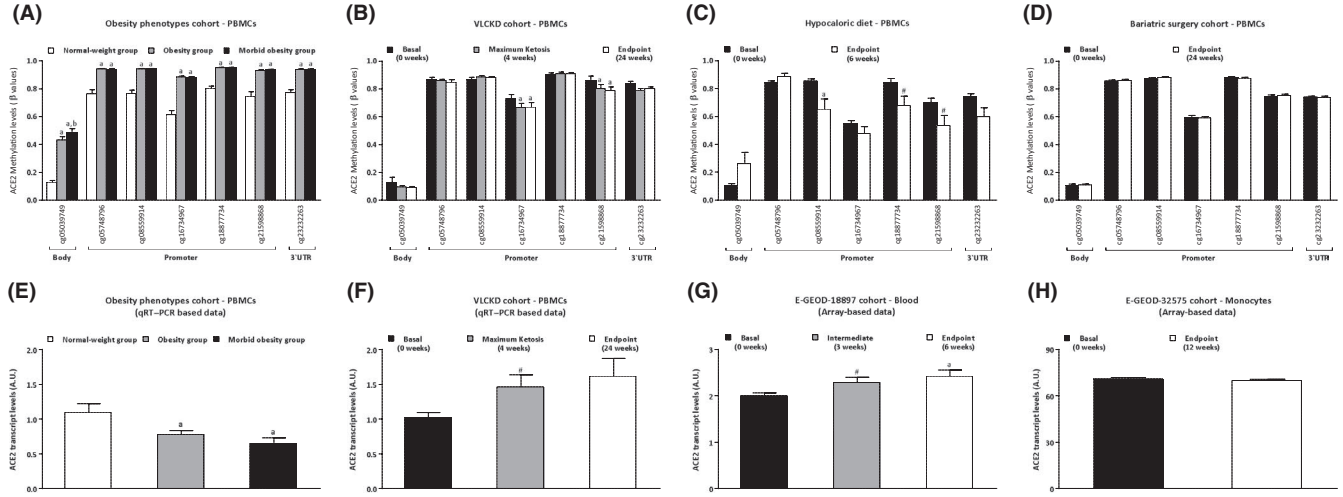

FIGURE 2 Differential methylation and expression levels of *ACE2* gene in peripheral blood mononuclear cells (PBMCs). *ACE2* DNA methylation levels: (A) based on the obesity phenotypes ($n = 10$, normal weight group; $n = 13$, obesity group; $n = 11$, morbid obesity group); (B) after following a very-low-calorie ketogenic diet (VLCKD, $n = 10$); (C) after following a hypocaloric diet ($n = 10$); (D) after bariatric surgery ($n = 24$). ACE2 transcript levels: (E) based on the obesity phenotypes ($n = 15$, normal weight group; $n = 15$, obesity group; $n = 15$, morbid obesity group); (F) after following a very-low-calorie ketogenic diet (VLCKD, $n = 10$); (G) after following a hypocaloric diet ($n = 12$); (H) after bariatric surgery ($n = 18$). Data show mean ± SEM (standard error of the mean). [a]denotes statistically significant differences when compared with those in the normal weight group as evaluated using ANOVA and statistically significant differences when compared basal levels as evaluated using repeated measures ANOVA, in all of them using a Bonferroni post hoc analysis. [#]denotes a trend towards statistical significance when compared basal levels as evaluated using repeated measures ANOVA

adiposity in both the transversal and longitudinal studies. To further evaluate the biological significance of the differential methylation observed in *ACE2* associated with obesity, a correlation analysis was performed between the methylation levels of the identified CpG sites and BMI in VAT and PBMCs samples. We found that higher β-values of the identified CpGs in VAT (Figure 4A,B) and PBMCs (Figure 4C–F) were correlated with higher BMI.

4 | DISCUSSION

The current study reveals a global obesity-related increase in methylation levels through the sequence of *ACE2* gene, which encodes the main SARS-CoV-2 entry factor,[6] in VAT that can be reflected in easily obtainable samples of PBMCs, but not in SAT compared with normal weight. This finding was independently of the presence of the obesity comorbidities, such as insulin resistance or NASH. Relevantly, the obesity-related methylation pattern was reversed after following a nutritional weight loss therapy, but not after bariatric surgery. Moreover, two CpG sites at the promoter of the *ACE2* gene, cg16734967 and cg21598868, were identified as potential biomarkers for monitoring obesity-related COVID-19 risk and its progression in a noninvasive sample (blood leukocytes). These results could be useful to clinicians for identifying subjects with susceptibility to SARS-CoV-2 infection and severity of COVID-19, as well as allowing personalization of treatment[17] by measuring ACE2 methylation levels in a noninvasive sample.

The high methylation levels in the regulatory region of *ACE2* observed in this study are associated with downregulation of the gene expression in VAT and PBMCs. This was concordant with a previous study that demonstrated an association between lower AT *ACE2* expression and adverse cardiometabolic health indices, including type 2 diabetes and obesity status.[7] VAT plays a relevant role in the onset of obesity comorbidities and also has led to interest in understanding the pathology of COVID-19 in patients with obesity.[40] However, other reports have evidenced an increased gene expression of ACE2 in other adipose tissue depots such as epicardial adipose tissue.[41]

Since the onset of the COVID-19 pandemic, several factors have been associated with a higher risk of infection and severity of the disease, such as age, sex, smoking and excess body weight. Epigenetic regulation has been proposed as a promising mechanistic target for understanding the physiopathology of SARS-CoV-2 infection and for developing epigenetic drugs for treatment and prevention.[42] DNA methylation levels in *ACE2* have been associated with increased vulnerability to COVID-19.[17,42] However, some studies have observed that under-methylation and over-methylation of *ACE2* in different tissues are also risk factors for COVID-19, underscoring the necessity for further investigation in this field.[17]

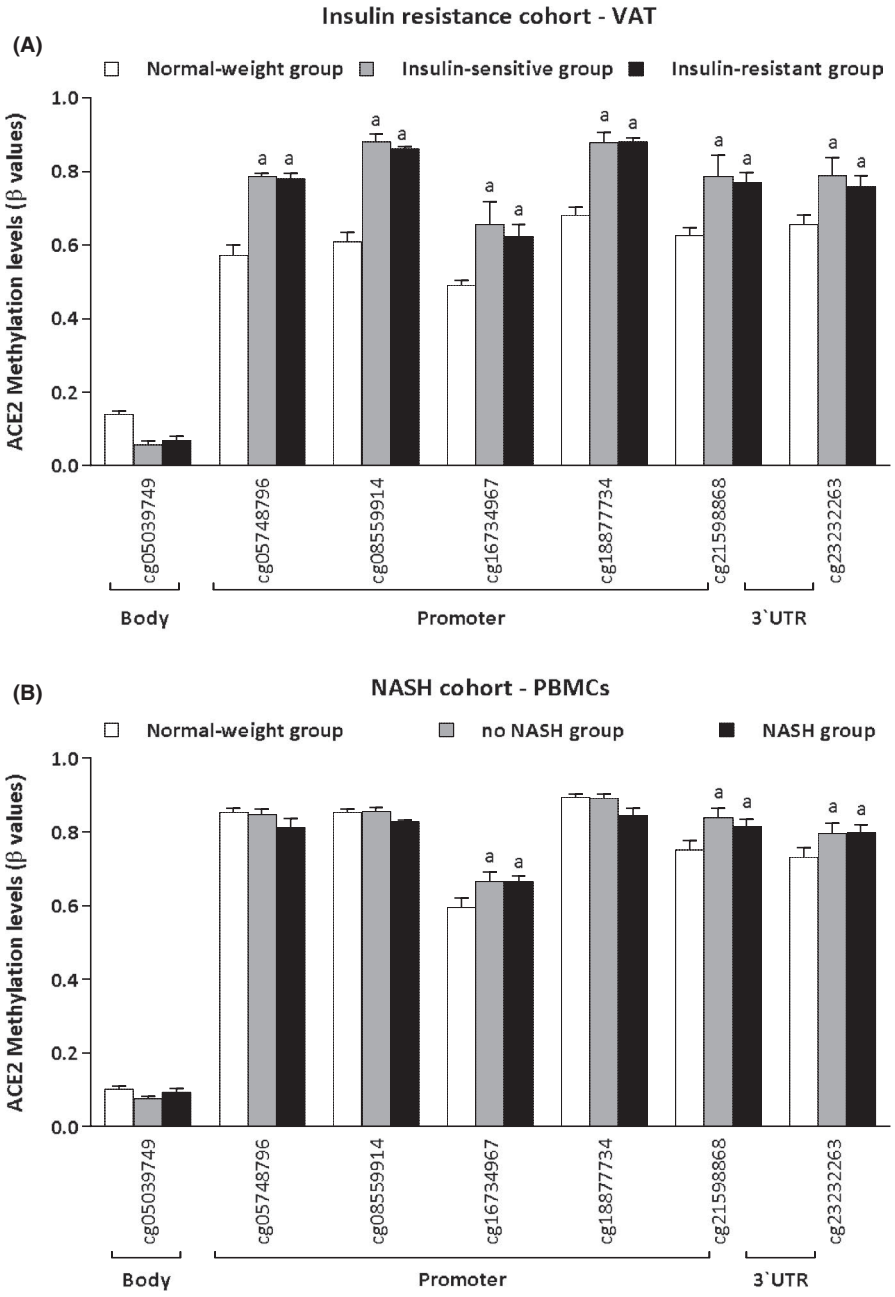

FIGURE 3 Differential *ACE2* methylation levels based on obesity-related comorbidities. (A) DNA methylation levels in visceral adipose tissue (VAT) comparing insulin-sensitive ($n = 5$) and insulin-resistant ($n = 7$) patients with obesity. (B) DNA methylation levels in peripheral blood mononuclear cells (PBMCs) based on nonalcholic steatohepatitis (NASH) status in individuals with obesity ($n = 10$, no NASH; $n = 10$, NASH). Data shown are mean ± SEM (standard error of the mean). [a]denotes statistically significant differences when compared with those in the normal weight group as evaluated using ANOVA and a Bonferroni post hoc analysis

It is known that dysfunctional AT secretes proinflammatory factors that promote disease development.[43-45] Our research group has previously proposed that increased health risk due to dysfunctional AT effect could be mediated by epigenetic mechanisms.[27,46] Similar mechanisms could be proposed for the contribution of AT to COVID-19 clinical severity. Epigenetic changes occurring in the VAT of obesity groups could lead to increased inflammation. Therefore, the results observed in this study could be related to the high severity of the COVID-19 disease observed in patients with obesity. This is related to the fact that the proinflammatory status promoted by the dysfunctional AT has been linked to the cytokine storm involved in the severity of COVID-19 disease.[7,47,48] Moreover, in the current study, the differential methylation levels observed in obesity compared with normal weight status were independent of obesity comorbidities such as insulin resistance or NASH. This is in agreement with a recent study demonstrating that excess body weight is significantly associated with severe forms of the disease, independent of its classical associated comorbidities.[1]

Because ACE2 is a crucial factor for SARS-CoV-2 infection and AT was proposed as a reservoir of the virus, our results could be counterintuitive. However, it was suggested that ACE2 downregulation induced by viral invasion may be especially detrimental in people with baseline ACE2 deficiency associated with the above conditions because it decreases the conversion of angiotensin II into angiotensin-(1-7), which

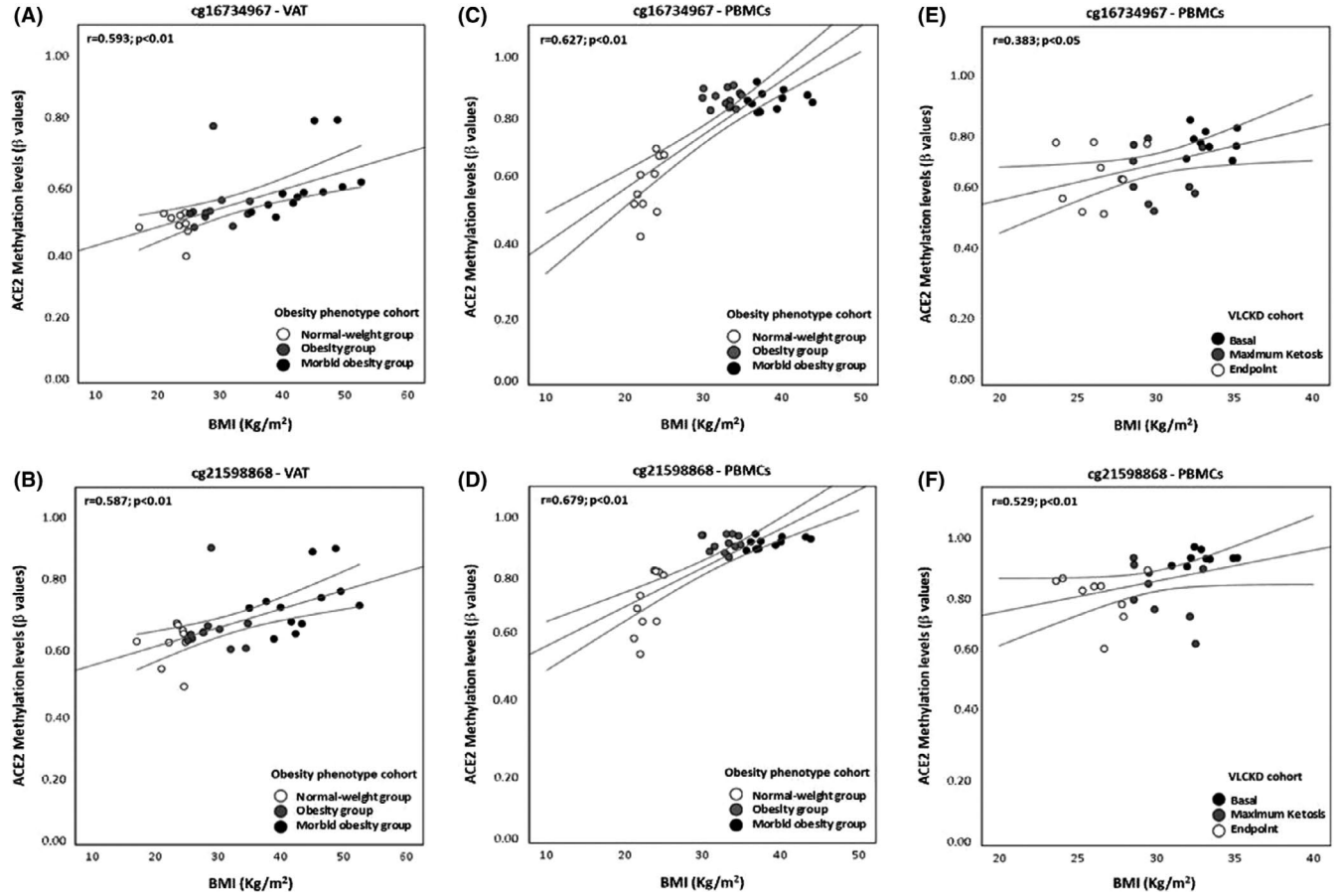

FIGURE 4 Scatter plot representing the correlation between *ACE2* methylation levels and body mass index (BMI). (A–D) Correlation between BMI and cg16734967 or cg21598868 methylation levels in visceral adipose tissue (VAT) and peripheral blood mononuclear cells (PBMCs) based on obesity phenotypes. (E–F) Correlation between BMI and cg16734967 or cg21598868 methylation levels in peripheral blood mononuclear cells (PBMCs) following a very-low-calorie ketogenic diet (VLCKD). The centre line represents the linear regression trend line. The lines above and below the centre line represent the upper and lower bounds of the 95% confidence interval around the trend line. *r*, Correlation coefficient evaluated using the Pearson test; *p*, *p*-value

has protective properties.[49] Thus, the proinflammatory status induced by the dysfunctional AT in obesity could promote the downregulation of ACE2 gene expression by increasing DNA methylation. Consequently, local angiotensin II accumulation could favour a vicious proinflammatory circle, thus promoting the cytokine storm.[50-52]

Considering the consistent association between obesity and COVID-19, strategies that can reverse the molecular factors involved in the risk and progression of infection are urgently needed. Weight loss is a therapeutic strategy for reversing the metabolic and molecular factors involved in the pathophysiology of obesity, such as reducing visceral fat mass and preserving muscle mass and function,[53-55] as well as decreasing inflammation and oxidative stress[12,14,25,34,56-58] or protumoral status.[35] In addition, weight loss therapies are also able to modulate the obesity-related methylation profile.[28,30,59,60] In line with these results, weight loss therapies based dietary energy restriction were able to reverse the obesity-related methylation levels of ACE2 with a putative increase in the transcript levels. However, after 6 months of bariatric surgery, the methylation and transcript levels of ACE2 did no change respect to baseline, even though patients showed a weight loss higher than 20%. It could be due to patients still remained in an excess body weight status (BMI > 25) or a potential metabolic stress observed in patients underwent this surgical intervention.[12,29]

Therefore, the methylation marks of *ACE2* gene in AT could provide useful tools for identifying at-risk individuals and predicting disease progression. However, obtaining an AT biopsy from a healthy subject is difficult without surgery. Instead of tissue biopsy, blood leukocytes are a suitable, minimally invasive sample for evaluating gene expression regulation and epigenetic markers.[61] The lack of reproduction in PBMCs of differences in all CpG sites observed in VAT could be a limitation to use PBMCs. It is because epigenetic mechanisms are tissue specific. However, despite considering that limitation, leukocytes

can be used as a surrogate to evaluate those CpG sites that are reproduced in both tissues as representative. In this context, the strength of the current work is that it adds relevant information by mirroring most of the *ACE2* methylation profile of VAT in PBMCs.

Possible limitations of the current study warrant consideration before interpretation of the findings. The study design does not allow us to demonstrate causation. Patients with normal weight in this cohort were older than patients with obesity and morbid obesity. Methylation levels are modulated with age,[62] and the results could be confounded by this fact. However, we did not find a correlation between global methylation levels of *ACE2* and age, suggesting that the results are independent of the differences in age between groups. Moreover, the sample size of this study could be considered small. However, the statistical significance found when using small populations usually indicates that there is a real difference between the experimental groups. These limitations do not invalidate the results but they reinforce the necessity to further evaluate the potential regulation of ACE2 expression by epigenetic mechanisms in preclinical models and to identify potential therapeutic targets and biomarkers for preventing and monitoring COVID-19 in patients with high risk such as patients with obesity.

In conclusion, the findings of this study demonstrate a methylation-based epigenetic regulation of the *ACE2* gene in VAT from patients with obesity. The obesity-related methylation profile is mirrored in PBMCs and reversed after a dietary-based weight loss therapy. It suggests that the association between obesity and predisposition to COVID-19 severity could be mediated by an epigenetic regulation of *ACE2* gen in VAT and its methylation levels measured in PBMCs could be a good biomarker for monitoring the management of COVID-19 in at-risk patients. These results warrant further research in larger cohorts of healthy controls and SARS-CoV2 infected subjects.

ACKNOWLEDGEMENTS

The authors thank all participants in this study and the research group involved in the project, as well as the individuals who performed the field work. Appreciation is also extended to Dr. Ignacio Sajoux, Medical Director at Pronokal Group, Barcelona, Spain, for financially supporting the longitudinal study of individuals on the very-low-calorie ketogenic diet (VLCKD). This work was supported by the PronoKal Group® and grants from the Fondo de Investigacion Sanitaria, as well as PI17/01287, PI20/00650, PI20/00628 and CP17/00088 research projects and CIBERobn from the Instituto de Salud Carlos III (ISCIII)-Subdireccion General de Evaluacion y Fomento de la Investigacion; Fondo Europeo de Desarrollo Regional (FEDER) and by Xunta de Galicia-GAIN (IN607B2020/09). Ana B Crujeiras is funded by a research contract 'Miguel Servet' (CP17/00088) from the ISCIII, co-financed by the European Regional Development Fund (FEDER). Manuel Macias-Gonzalez was the recipient of the Nicolas Monardes Program from the 'Servicio Andaluz de Salud, Junta de Andalucía', Spain (RC-0001-2018 and C-0029-2014). The funders had no involvement in the study design or interpretation of the results.

CONFLICT OF INTEREST

All authors declare to have no competing financial interests in relation to the work described except A.B.C., D. dL and F.F.C. who received advisory board fees and/or research grants from Pronokal Protein Supplies, Spain.

AUTHOR CONTRIBUTIONS

ABC and AGI designed and performed the experiments, analysed the data and wrote the manuscript; MMG, DL, JMFR, JMN, JAM, CN and CN recruited and followed up the patients; MCC and MM-G helped with data analysis; PO, MPP, JAM, JMFR, MAMO and FFC contributed to the interpretation of data and the discussion; FFC and ABC obtained funding. All authors read and approved the final manuscript.

ORCID

Ana B. Crujeiras https://orcid.org/0000-0003-4392-0301

REFERENCES

1. Pietri L, Giorgi R, Bégu A, et al. Excess body weight is an independent risk factor for severe forms of COVID-19. *Metabolism*. 2021;117:154703.
2. Rottoli M, Bernante P, Belvedere A, et al. How important is obesity as a risk factor for respiratory failure, intensive care admission and death in hospitalised COVID-19 patients? Results from a single Italian centre. *Eur J Endocrinol*. 2020;183(4):389-397.
3. Stefan N, Birkenfeld AL, Schulze MB. Global pandemics interconnected obesity, impaired metabolic health and COVID-19. *Nat Rev Endocrinol*. 2021;17(3):135-149.
4. Goossens GH, Dicker D, Farpour-Lambert NJ, et al. Obesity and COVID-19: a perspective from the European Association for the Study of Obesity on Immunological Perturbations, Therapeutic Challenges, and Opportunities in Obesity. *Obes Facts*. 2020;13(4):439-452.
5. Post A, Bakker SJL, Dullaart RPF. Obesity, adipokines and COVID-19. *Eur J Clin Invest*. 2020;50(8):e13313.
6. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*. 2020;181(2):271-280.e8.
7. El-Sayed Moustafa JS, Jackson AU, Brotman SM, et al. ACE2 expression in adipose tissue is associated with COVID-19 cardiometabolic risk factors and cell type composition. *medRxiv* Prepr Serv Heal Sci. 2020;2020.08.11.20171108.

8. Warner FJ, Rajapaksha H, Shackel N, Herath CB. ACE2: from protection of liver disease to propagation of COVID-19. *Clin Sci*. 2020;134(23):3137-3158.
9. Sharma RK, Li J, Krishnan S, Richards EM, Raizada MK, Mohandas R. Angiotensin-converting enzyme 2 and COVID-19 in cardiorenal diseases. *Clin Sci*. 2021;135(1):1-17.
10. Cao X, Song LN, Yang JK. ACE2 and energy metabolism: the connection between COVID-19 and chronic metabolic disorders. *Clin Sci*. 2021;135(3):535-554.
11. Giordano A, Frontini A, Cinti S. Convertible visceral fat as a therapeutic target to curb obesity. *Nat Rev Drug Discov*. 2016;15(6):405-424.
12. Crujeiras AB, Gomez-Arbelaez D, Zulet MA, et al. Plasma FGF21 levels in obese patients undergoing energy-restricted diets or bariatric surgery: a marker of metabolic stress? *Int J Obes (Lond)*. 2017;41(10):1570-1578.
13. López-Domènech S, Martínez-Herrera M, Abad-Jiménez Z, et al. Dietary weight loss intervention improves subclinical atherosclerosis and oxidative stress markers in leukocytes of obese humans. *Int J Obes (Lond)*. 2019;43(11):2200-2209.
14. Carreira MC, Izquierdo AG, Amil M, Casanueva FF, Crujeiras AB. Oxidative stress induced by excess of adiposity is related to a downregulation of hepatic SIRT6 expression in obese individuals. *Oxid Med Cell Longev*. 2018;2018:6256052.
15. Chlamydas S, Papavassiliou AG, Piperi C. Epigenetic mechanisms regulating COVID-19 infection. *Epigenetics*. 2021;16(3):263-270.
16. DE Freitas NL, Azevedo PRG, BrandÃo F. A glance upon Epigenetic and COVID-19. *An Acad Bras Cienc*. 2020;92(4):e20201451.
17. Ragia G, Manolopoulos VG. Assessing COVID-19 susceptibility through analysis of the genetic and epigenetic diversity of ACE2-mediated SARS-CoV-2 entry. *Pharmacogenomics*. 2020;21(18):1311-1329.
18. AbdelHamid SG, Refaat AA, Benjamin AM, et al. Deciphering epigenetic(s) role in modulating susceptibility to and severity of COVID-19 infection and/or outcome: a systematic rapid review. *Environ Sci Pollut Res Int*. 2021;1-13.
19. Pontecorvi G, Bellenghi M, Ortona E, Carè A. microRNAs as new possible actors in gender disparities of Covid-19 pandemic. *Acta Physiol*. 2020;230(1):e13538.
20. Elemam NM, Hasswan H, Aljaibeji H, Sulaiman N. Circulating soluble ace2 and upstream microrna expressions in serum of type 2 diabetes mellitus patients. *Int J Mol Sci*. 2021;22(10):5263.
21. Crujeiras AB, Diaz-Lagares A, Sandoval J, et al. DNA methylation map in circulating leukocytes mirrors subcutaneous adipose tissue methylation pattern: a genome-wide analysis from non-obese and obese patients. *Sci Rep*. 2017;7:41903.
22. Díaz-Rúa R, Keijer J, Caimari A, van Schothorst EM, Palou A, Oliver P. Peripheral blood mononuclear cells as a source to detect markers of homeostatic alterations caused by the intake of diets with an unbalanced macronutrient composition. *J Nutr Biochem*. 2015;26(4):398-407.
23. Caimari A, Oliver P, Rodenburg W, Keijer J, Palou A. Slc27a2 expression in peripheral blood mononuclear cells as a molecular marker for overweight development. *Int J Obes (Lond)*. 2010;34(5):831-839.
24. Reynés B, García-Ruiz E, Palou A, Oliver P. The intake of high-fat diets induces an obesogenic-like gene expression profile in peripheral blood mononuclear cells, which is reverted by dieting. *Br J Nutr*. 2016;115(11):1887-1895.
25. Crujeiras AB, Parra D, Milagro FI, et al. Differential expression of oxidative stress and inflammation related genes in peripheral blood mononuclear cells in response to a low-calorie diet: a nutrigenomics study. *OMICS*. 2008;12(4):251-261.
26. Crujeiras AB, Parra D, Goyenechea E, Martínez JA. Sirtuin gene expression in human mononuclear cells is modulated by caloric restriction. *Eur J Clin Invest*. 2008;38(9):672-678.
27. Crujeiras AB, Diaz-Lagares A, Moreno-Navarrete JM, et al. Genome-wide DNA methylation pattern in visceral adipose tissue differentiates insulin-resistant from insulin-sensitive obese subjects. *Transl Res*. 2016;178:13-24.e5.
28. Nicoletti CF, Cortes-Oliveira C, Noronha NY, et al. DNA methylation pattern changes following a short-term hypocaloric diet in women with obesity. *Eur J Clin Nutr*. 2020;74(9):1345-1353.
29. Nicoletti CF, Pinhel MAS, Diaz-Lagares A, et al. DNA methylation screening after roux-en Y gastric bypass reveals the epigenetic signature stems from genes related to the surgery per se. *BMC Med Genomics*. 2019;12(1):72.
30. Crujeiras AB, Izquierdo AG, Primo D, et al. Epigenetic landscape in blood leukocytes following ketosis and weight loss induced by a very low calorie ketogenic diet (VLCKD) in patients with obesity. *Clin Nutr*. 2021;40(6):3959-3972.
31. Sandoval J, Heyn H, Moran S, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics*. 2011;6(6):692-702.
32. Moran S, Arribas C, Esteller M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. *Epigenomics*. 2016;8(3):389-399.
33. Simera I, Moher D, Hoey J, Schulz KF, Altman DG. A catalogue of reporting guidelines for health research. *Eur J Clin Invest*. 2010;40(1):35-53.
34. Izquierdo AG, Carreira MC, Rodriguez-Carnero G, et al. Weight loss normalizes enhanced expression of the oncogene survivin in visceral adipose tissue and blood leukocytes from individuals with obesity. *Int J Obes*. 2021;45(1):206-216.
35. Izquierdo AG, Carreira MC, Amil M, et al. An energy restriction-based weight loss intervention is able to reverse the effects of obesity on the expression of liver tumor-promoting genes. *FASEB J*. 2020;34(2):2312-2325.
36. Lorenzo PM, Izquierdo AG, Diaz-Lagares A, et al. ZNF577 Methylation levels in leukocytes from women with breast cancer is modulated by adiposity, Menopausal State, and the Mediterranean Diet. *Front Endocrinol (Lausanne)*. 2020;11:245.
37. Crujeiras AB, Morcillo S, Diaz-Lagares A, et al. Identification of an episignature of human colorectal cancer associated with obesity by genome-wide DNA methylation analysis. *Int J Obes*. 2019;43(1):176-188.
38. Crujeiras AB, Diaz-Lagares A, Stefansson OA, et al. Obesity and menopause modify the epigenomic profile of breast cancer. *Endocr Relat Cancer*. 2017;24(7):351-363.
39. MacLaren RE, Cui W, Lu H, Simard S, Cianflone K. Association of adipocyte genes with ASP expression: a microarray analysis of subcutaneous and omental adipose tissue in morbidly obese subjects. *BMC Med Genomics*. 2010;3:3.
40. Verdecchia P, Cavallini C, Spanevello A, Angeli F. The pivotal link between ACE2 deficiency and SARS-CoV-2 infection. *Eur J Intern Med*. 2020;76:14-20.

41. Couselo-Seijas M, Almengló C, Agra-Bermejo M, et al. Higher ACE2 expression levels in epicardial cells than subcutaneous stromal cells from patients with cardiovascular disease: diabetes and obesity as possible enhancer. *Eur J Clin Invest*. 2021;51(5):e13463.
42. Sen R, Garbati MR, Bryant K, Lu Y. Epigenetic mechanisms influencing COVID-19. *Genome*. 2021;64(4):372-385.
43. Carobbio S, Pellegrinelli V, Vidal-Puig A. Adipose tissue function and expandability as determinants of lipotoxicity and the metabolic syndrome. *Adv Exp Med Biol*. 2017;960:161-196.
44. Unamuno X, Gómez-Ambrosi J, Rodríguez A, Becerril S, Frühbeck G, Catalán V. Adipokine dysregulation and adipose tissue inflammation in human obesity. *Eur J Clin Invest*. 2018;48(9):e12997.
45. Blüher M. Adipose tissue inflammation: A cause or consequence of obesity-related insulin resistance? *Clin Sci*. 2016;130(18):1603-1614.
46. Cabia B, Andrade S, Carreira MC, Casanueva FF, Crujeiras AB. A role for novel adipose tissue-secreted factors in obesity-related carcinogenesis. *Obes Rev*. 2016;17(4):361-376.
47. Malavazos AE, Corsi Romanelli MM, Bandera F, Iacobellis G. Targeting the Adipose Tissue in COVID-19. *Obesity (Silver Spring)*. 2020;28(7):1178-1179.
48. Ryan PM, Caplice NM. Is adipose tissue a reservoir for viral spread, immune activation, and cytokine amplification in coronavirus disease 2019? *Obesity (Silver Spring)*. 2020;28(7):1191-1194.
49. Krams IA, Luoto S, Rantala MJ, Jõers P, Krama T. Covid-19: fat, obesity, inflammation, ethnicity, and sex differences. *Pathogens*. 2020;9(11):887.
50. Oudit GY, Kassiri Z, Jiang C, et al. SARS-coronavirus modulation of myocardial ACE2 expression and inflammation in patients with SARS. *Eur J Clin Invest*. 2009;39(7):618-625.
51. Artom N, Montecucco F, Mach F, Dallegri F, Pende A. Angiotensin II receptor antagonists in acute coronary syndromes. *Eur J Clin Invest*. 2014;44(2):219-230.
52. da Silva AR, Fraga-Silva RA, Stergiopulos N, Montecucco F, Mach F. Update on the role of angiotensin in the pathophysiology of coronary atherothrombosis. *Eur J Clin Invest*. 2013;43(12):1328-1338.
53. Casanueva FF, Castellana M, Bellido D, et al. Ketogenic diets as treatment of obesity and type 2 diabetes mellitus. *Rev Endocr Metab Disord*. 2020;21(3):381-397.
54. Sajoux I, Lorenzo PM, Gomez-Arbelaez D, et al. Effect of a very-low-calorie ketogenic diet on circulating myokine levels compared with the effect of bariatric surgery or a low-calorie diet in patients with obesity. *Nutrients*. 2019;11(10):2368.
55. Gomez-Arbelaez D, Bellido D, Castro AI, et al. Body composition changes after very-low-calorie ketogenic diet in obesity evaluated by 3 standardized methods. *J Clin Endocrinol Metab*. 2017;102(2):488-498.
56. de Luis D, Domingo JC, Izaola O, Casanueva FF, Bellido D, Sajoux I. Effect of DHA supplementation in a very low-calorie ketogenic diet in the treatment of obesity: a randomized clinical trial. *Endocrine*. 2016;54(1):111-122.
57. Crujeiras AB, Parra D, Goyenechea E, Abete I, Martínez JA. Tachyphylaxis effects on postprandial oxidative stress and mitochondrial-related gene expression in overweight subjects after a period of energy restriction. *Eur J Nutr*. 2009;48(6):341-347.
58. Goyenechea E, Parra D, Crujeiras AB, Abete I, Martínez JA. A nutrigenomic inflammation-related PBMC-based approach to predict the weight-loss regain in obese subjects. *Ann Nutr Metab*. 2009;54(1):43-51.
59. Nicoletti CF, Pinhel MS, Noronha NY, Jácome A, Crujeiras AB, Nonino CB. Association of MFSD3 promoter methylation level and weight regain after gastric bypass: Assessment for 3 y after surgery. *Nutrition*. 2020;70:110499.
60. Fraszczyk E, Luijten M, Spijkerman AMW, et al. The effects of bariatric surgery on clinical profile, DNA methylation, and ageing in severely obese patients. *Clin Epigenetics*. 2020;12(1):14.
61. Keller M, Yaskolka Meir A, Bernhart SH, et al. DNA methylation signature in blood mirrors successful weight-loss during lifestyle interventions: the CENTRAL trial. *Genome Med*. 2020;12(1):97.
62. Heyn H, Li N, Ferreira HJ, et al. Distinct DNA methylomes of newborns and centenarians. *Proc Natl Acad Sci USA*. 2012;109(26):10522-10527.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Izquierdo AG, Carreira MC, Boughanem H, et al. Adipose tissue and blood leukocytes ACE2 DNA methylation in obesity and after weight loss. *Eur J Clin Invest*. 2022;52:e13685. doi:10.1111/eci.13685