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The APOA1BP-SREBF-NOTCH axis isassociated with reduced atherosclerosis risk in morbidly obese patients
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Corresponding Author:	José Manuel Fernández-Real Hospital Dr.Josep Trueta Girona, Girona SPAIN
First Author:	Jordi Mayneris-Perxachs
Order of Authors:	Jordi Mayneris-Perxachs
	Josep Puig
	Rémy Burcelin
	Marc-Emmanuel Dumas
	Richard H. Barton
	Lesley Hoyles
	José Manuel Fernández-Real
	Massimo Federici
Abstract:	Background & Aims: Atherosclerosis is characterized by an inflammatory disease linked to excessive lipid accumulation in the artery wall. The Notch signalling pathway has been shown to play a key regulatory role in the regulation of inflammation. Recently, in vitro and pre-clinical studies have shown that apolipoprotein A-I binding protein (AIBP) regulates cholesterol metabolism(SREBP) and NOTCH signalling (haematopoiesis) and may be protective against atherosclerosis, but the evidence in humans is scarce. Methods: Weevaluated the APOA1BP-SREBF-NOTCH axis in association with atherosclerosis in two well-characterized cohorts of morbidly obese patients (n = 78) within the FLORINASH study, including liver transcriptomics, 1 H-NMR plasma metabolomics, high-resolution ultrasonography evaluating carotid intima-media thickness (cIMT), and haematological parameters. Results: The liver expression levels of APOA1BP were associated with lower cIMT and leukocyte counts, a better plasma lipid profile and higher circulating levels of metabolites associated with lower risk of atherosclerosis (glycine, histidine and asparagine). Conversely, liver SREBF and NOTCH mRNAs were positively associated with atherosclerosis, liver steatosis, an unfavourable lipid profile, higher leukocytes and increased levels of metabolites linked to inflammation and CVD such as branched-chain amino acids and glycoproteins. APOA1BP and NOTCH signalling also had a strong association, as revealed by the negative correlations among APOA1BP expression levels and those of all NOTCH receptors and jagged ligands. Conclusions: We hereprovide the first evidence in human liverof the putative APOA1BP - SREBF-NOTCH axis signalling pathway and its association with atherosclerosis and inflammation.

The *APOA1BP-SREBF-NOTCH* axis is associated with reduced atherosclerosis risk in morbidly obese patients

Jordi Mayneris-Perxachs[1,2], Josep Puig[1], Rémy Burcelin[3,4], Marc-Emmanuel Dumas[5,6], Richard H. Barton[5], Lesley Hoyles[7], Massimo Federici[8], José-Manuel Fernández-Real[1,2*]

[1] Department of Endocrinology, Diabetes and Nutrition, Hospital of Girona "Dr Josep Trueta", Departament de Ciències Mèdiques, University of Girona, Girona Biomedical Research Institute (IdibGi), Girona, Spain

[2] CIBERobn Pathophysiology of Obesity and Nutrition, Instituto de Salud Carlos III, Madrid, Spain

[3] Institut National de la Santé et de la Recherche Médicale (INSERM), Toulouse, France

[4] Université Paul Sabatier (UPS), Unité Mixte de Recherche (UMR) 1048, Institut des Maladies Métaboliques et Cardiovasculaires (I2MC), Team 2: 'Intestinal Risk Factors, Diabetes, Dyslipidemia, and Heart Failure' F-31432 Toulouse Cedex 4, France

[5] Section of Computational and Systems Medicine, Division of Integrative Systems Medicine, Department of Metabolism, Digestion and Reproduction, Imperial College London, Exhibition Road, London SW7 2AZ, United Kingdom

[6] Section of Genomic and Environmental Medicine, National Heart & Lung Institute, Imperial College London, Dovehouse Street, SW3 6KY London, United Kingdom

[7] Department of Biosciences, Nottingham Trent University, Clifton Campus, Nottingham NG11 8NS, United Kingdom

[8] Department of Systems Medicine, University of Rome Tor Vergata, Via Montpellier 1 00133, Rome, Italy

Corresponding author:

José-Manuel Fernández-Real, M.D., PhD.

Department of Diabetes, Endocrinology and Nutrition.

Dr. Josep Trueta University Hospital. Girona Biomedical Research Institute (IdIBGi).

Carretera de França s/n, 17007, Girona, Spain.

Phone: +34 972940200 / Fax: +34 97294027.

E-mail: jmfreal@idibgi.org

Running title: *APOA1BP-SREBF-NOTCH* axis and atherosclerosis risk

Keywords: apolipoprotein A1 binding protein, *APOA1BP, NOTCH, SREBF,* atherosclerosis, inflammation, haematopoiesis, liver

Abbreviations

AAV, adeno-associated virus; ABCA1, ATP-binding cassette transporter A1; AIBP, apolipoprotein A-I binding protein; APOA1, apolipoprotein A1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCAA, branched-chain amino acids; CCA; common carotid arteries; cIMT, carotid intima-media thickness; CAD, coronary artery disease; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HPSC, hematopoietic progenitor and stem cells; HsCRP, high sensitive C-reactive protein; ICA, internal carotid arteries; LCAT, lecithin-cholesterol acyltransferase; MWAS, metabolite-wide association studies; NAG, N-acetylglycoproteins; O-PLS, orthogonal partial least squares; PCA, principal component analysis; preCCA, pre bifurcation common carotid arteries; proCCA, proximal segment common carotid arteries; RBC, red blood cells; RCT, reverse cholesterol transport; SR-BI; scavenger receptor class B member 1; TG, triglycerides; WBC, white blood cells

Abstract

Background & Aims: Atherosclerosis is characterized by an inflammatory disease linked to excessive lipid accumulation in the artery wall. The Notch signalling pathway has been shown to play a key regulatory role in the regulation of inflammation. Recently, *in vitro* and pre-clinical studies have shown that apolipoprotein A-I binding protein (AIBP) regulates cholesterol metabolism (SREBP) and NOTCH signalling (haematopoiesis) and may be protective against atherosclerosis, but the evidence in humans is scarce.

Methods: We evaluated the *APOA1BP-SREBF-NOTCH* axis in association with atherosclerosis in two well-characterized cohorts of morbidly obese patients (*n* = 78) within the FLORINASH study, including liver transcriptomics, ^{1}H-NMR plasma metabolomics, high-resolution ultrasonography evaluating carotid intima-media thickness (cIMT), and haematological parameters.

Results: The liver expression levels of *APOA1BP* were associated with lower cIMT and leukocyte counts, a better plasma lipid profile and higher circulating levels of metabolites associated with lower risk of atherosclerosis (glycine, histidine and asparagine). Conversely, liver *SREBF* and *NOTCH* mRNAs were positively associated with atherosclerosis, liver steatosis, an unfavourable lipid profile, higher leukocytes and increased levels of metabolites linked to inflammation and CVD such as branched-chain amino acids and glycoproteins. *APOA1BP* and NOTCH signalling also had a strong association, as revealed by the negative correlations among *APOA1BP* expression levels and those of all NOTCH receptors and jagged ligands.

77   **Conclusions:** We here provide the first evidence in human liver of the putative

78   *APOA1BP-SREBF-NOTCH* axis signalling pathway and its association with

79   atherosclerosis and inflammation.

Atherosclerosis is the major cause of cardiovascular disease (CVD), the leading cause of death worldwide. Several studies have shown the anti-atherogenic potential of the high-density lipoprotein (HDL)-mediated reverse cholesterol transport (RCT) [1]. In this process, excess cholesterol is transported from macrophages and peripheral tissues back to the liver for excretion. A critical part of RCT is cholesterol efflux, in which intracellular cholesterol is released and collected by apolipoprotein A1 (APOA1), the major component of HDL [2]. In fact, the cholesterol efflux potential of HDL is a better predictor of CVD than circulating HDL levels [3]. The ATP-binding cassette transporter A1 (ABCA1), a cell-membrane protein, is the major transporter that mediates this assembly of cholesterol with APOA1, which is the rate-limiting step in the formation of nascent HDLs.

The APOA1 binding protein (AIBP), encoded by *APOA1BP* in humans, is a secreted protein physically associated with APOA1 [4] that enhances cholesterol efflux *in vitro* from macrophages [5] and endothelial cells [6] in the presence of APOA1 or HDL, partly by facilitating apoA1 binding to ABCA1 and preventing ABCA1 degradation [5]. Recently, AIBP has shown to protect against atherosclerosis *in vivo* in $Apoe^{-/-}$ and $Ldlr^{-/-}$ mice by promoting cholesterol efflux and ameliorating inflammation [7,8]. Human *APOA1BP* mRNA is ubiquitously expressed and abundant in most human secretory organs, with the highest expression in kidney, heart, liver, thyroid gland, adrenal gland, and testis [4]. It is of note that kidney and liver are the major sites of APOA1 catabolism.

Atherosclerosis is characterized by progressive accumulation of lipids and leukocytes in the intima layer of the arterial wall. It is considered a chronic inflammation disease with monocytes/macrophages, the main immune cells of the innate immune response, playing a major role in all stages of atherosclerosis [9]. Notably, the Notch pathway is well-recognized as a major regulator of cell fate in stem cells and the differentiation of the

105 various cell types of the immune system [10]. Recently, the Notch signalling pathway

106 was shown to play an important role in the onset and progression of atherosclerosis by

107 promoting inflammation through induction of a pro-inflammatory M1 phenotype in

108 macrophages, a switch towards a Th-1 like inflammatory phenotype in differentiated

109 regulatory T cells, and promoting CD8 cytotoxic T cells [11].

110 Recent evidence suggests a control of hematopoietic progenitor and stem cells (HPSC)

111 by cholesterol pathways [12], thereby linking haematopoiesis with atherosclerosis.

112 Interestingly, a connection between AIBP-mediated cholesterol metabolism and Notch

113 signalling has been described recently. AIBP was shown to regulate Notch signalling

114 through relocalization of γ-secretase from lipid to non-lipid rafts in mice [13] and

115 zebrafish Aibp2-mediated cholesterol efflux was shown to active endothelial Srebp2,

116 which in turn trans-activates Notch and promotes HSPC emergence. These observations

117 suggest a role for the AIBP-SREBP-NOTCH axis in atherosclerosis [14]. Although the

118 bone marrow is the primary site of haematopoiesis in adults, the liver is the main site

119 during prenatal development and hepatic haematopoiesis is believed to persist into

120 adulthood [15,16].

121 No study has, to date, addressed the potential association between AIBP and Notch

122 signalling in humans. Here, we studied this connection in two well-characterized cohorts

123 of morbidly obese patients recruited to the FLORINASH study (17) combining hepatic

124 transcriptome, plasma metabolome, and carotid intima-media thickness (cIMT)

125 measures.

126

Subjects and Methods

STUDY POPULATION AND SAMPLE COLLECTION

The study population ($n = 78$) included two cohorts of morbidly obese patients aged 22 to 61 years old recruited at the Endocrinology Service of the Hospital Universitari de Girona Dr Josep Trueta (Girona, Spain, $n = 32$) and at the Center for Atherosclerosis of Policlinico Tor Vergata University of Rome (Rome, Italy, $n = 46$), as part of the FLORINASH study [17]. All subjects gave written informed consent, which was validated and approved by the ethical committees of the Hospital Universitari Dr Josep Trueta (Comitè d'Ètica d'Investigació Clínica, approval number 2009 046) and Policlinico Tor Vergata University of Rome (Comitato Etico Indipendente, approval number 28-05-2009). Inclusion criteria included Caucasian origin, stable body weight 3 months before the study, free of any infection one month preceding the study and absence of any systemic disease. Exclusion criteria included presence of liver disease and thyroid dysfunction, alcohol consumption (> 20 g/day), hepatitis B, drug-induced liver injury, and dyslipidaemia medication. Plasma samples were obtained during the week before elective gastric bypass surgery, during which a liver biopsy was sampled. All samples were stored at −80 °C. Liver samples were collected in RNAlater, fragmented and immediately flash frozen in liquid nitrogen before storage at −80 °C.

ANTHROPOMETRIC AND LABORATORY MEASUREMENTS

Blood pressure was measured using a blood pressure monitor (Hem-703C, Omron, Barcelona, Spain), with the subject seated and after 5 min of rest. Three readings were obtained and the mean value was used in the analyses. Waist circumference was measured with a soft tape midway between the lowest rib and the iliac crest. Obesity was considered as a body mass index (BMI) ≥ 30 kg/m^2.

After 8 h fasting, blood was drawn for the measurement of plasma lipids, glucose and

insulin. Glucose and lipids were determined by standard laboratory methods. Intra-assay

and inter-assay coefficients of variation (CV) were less than 4 %. Serum insulin was

measured in duplicate using a monoclonal immunoradiometric assay (Medgenix

Diagnostics, Fleunes, Belgium). The intra-assay CV was 5.2 % at a concentration of 10

mU/L and 3.4 % at 130 mU/L. The interassay CVs were 6.9 % and 4.5 % at 14 and 89

mU/L, respectively. Glycated haemoglobin (HbA1c) was measured by high-pressure

liquid chromatography using a fully automated glycated haemoglobin analyser system

(Hitachi L-9100; Hitachi, Tokyo, Japan). Insulin resistance was determined by the

homeostasis model assessment of insulin resistance (HOMA-IR). High sensitive C-

reactive protein (HsCRP) was determined by ultrasensitive assay (Beckman, Fullerton,

CA), with intra-and inter-assay CVs of 4 %. Serum alanine aminotransferase (ALT) and

aspartate aminotransferase (AST) levels were determined using enzymatic methods.

LIVER HISTOLOGY

Liver biopsies were analysed by a single pathologist expert in hepatic pathology. For each

liver sample, haematoxylin and eosin, reticulin, and Masson's trichrome staining were

performed. Hepatic steatosis grade was determined according to the scoring system for

NAFLD and defined as absent (grade 0: <5% steatosis), mild (grade 1: 5-33% steatosis),

moderate (grade 2: >33-66% steatosis) or severe (grade 3: >66% steatosis) [18]. Images

were independently evaluated by two radiologists blinded to clinical and laboratory data.

¹H NUCLEAR MAGNETIC RESONANCE (¹H-NMR) METABOLIC PROFILING

Metabolomics analyses have been previously described [17]. Briefly, plasma samples were thawed at room temperature and 350 μL aliquots were carefully placed in 5-mm NMR tubes. Then, 150 μL of saline solution (0.9 % NaCl) were added and the mixture was gently vortexed. Spectroscopic analyses were performed on a Bruker DRX600 spectrometer equipped with either a 5-mm TXI probe operating at 600.13 MHz or a 5-mm BBI probe operating at 600.44 MHz. Spectra were acquired using a water suppressed Carr-Purcell-Meiboom-Gill using the Bruker program *cpmgpr* (RD 90º-(τ-180º-τ) n-acquire). A RD of 2 s was employed for net magnetization relaxation, during which noise irradiation was applied in order to suppress the large water proton signal. A number of loops $n = 100$ and a spin-echo delay $τ = 400$ μs was used to allow spectral editing through T2 relaxation and therefore attenuation of broad signals. For each sample, 128 scans were recorded in 32K data points with a spectral with of 20 ppm. All NMR spectra were processed using Topspin (Bruker Biospin, UK). Free induction decays were multiplied by an exponential function corresponding to a line broadening of 0.3 Hz and Fourier transformed. Spectra were automatically phased, baseline-corrected and referenced to the anomeric doublet of glucose (5.23 ppm). The spectra were all then imported to MATLAB and the region around the water resonance ($δ=4.5-5.0$) was removed.

TRANSCRIPTOMICS

Transcriptomic analyses have been previously described [17]. Briefly, RNA from liver biopsy samples was extracted using standard extraction protocols (TRIzol) by Miltenyi Biotec as previously reported. RNA quality (gel images, RNA integrity number and electropherograms) was assessed using an Agilent 2100 Bioanalyzer platform (Agilent

Technologies). An RNA integrity number >6 was considered sufficient for gene expression experiments [19]. One-hundred ng of total RNA were used for linear T7-based amplification of RNA for each sample. cDNA was prepared by amplification of the RNA and labelled with Cy3 using the Agilent Low Input Quick Amp Labeling Kit according to the manufacturer's instructions. The amounts of cDNA and dye that were incorporated were measured by an ND-1000 spectrophotometer (NanoDrop Technologies). Hybridization of the Agilent Whole Human Genome Oligo Microarrays $4 \times 44K$ was done following the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization Kit. The fluorescence signals of the hybridized Agilent microarrays were detected using Agilent's Microarray Scanner after washing with Agilent Gene Expression Wash Buffer twice and with acetonitrile once. Feature intensities were determined using Agilent Feature Extraction Software. Microarray data were processed and normalized using R and the BioConductor package LIMMA (Linear Models for Microarray Data) [20]. Raw data quality was assessed using pseudoMA and box plots. A background correction was applied and normalization of the green channel between arrays was done using 'cyclicloess' between pairs of arrays. Control and low-expressed probes were removed and only those probes brighter than the negative controls ($\geq 10\%$) on at least one array were kept. Batch-corrected data were obtained using removeBatchEffect based on 'Batch' [20]. Probes with no associated gene ID were removed. Finally, data were averaged based on an association to a particular gene.

HIGH-RESOLUTION ULTRASONOGRAPHY CAROTID EVALUATION

222

223 Carotid arteries were assessed using a Siemens Acuson S2000 (Mochida Siemens

224 Medical System, Tokyo, Japan) ultrasound system with a 7.5 MHz linear array

225 transducer. Images were independently evaluated by two radiologists blinded to clinical

226 and laboratory data. cIMT values were manually measured in 12 carotid segments:

227 internal (i) and external walls (e) of the right and left common carotid arteries (CCA) in

228 a proximal segment (proCCA), in a plaque-free segment 10 mm from the bifurcation

229 (preCCA) and in the internal carotid arteries (ICA). For the left and right arteries, the

230 mean CCA and ICA values for each subject were calculated from these four

231 measurements (mRCCA and mLCCA) and two measurement (mRICA and mLICA),

232 respectively. The average of all measurements was reported as overall cIMT (mCA). Sub-

233 clinical atherosclerosis was defined as a cut-off value of overall cIMT >0.78 mm [21]. A

234 plaque was defined as a focal thickening ≥1.2 mm in any of 12 carotid segments (near

235 and far walls of the right and left common carotid arteries, bifurcation and internal carotid

236 artery).

237

STATISTICAL ANALYSES

238

239 *Univariate statistics.* Data distribution and normality of variables were checked visually,

240 and using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Baseline characteristics are

241 presented as means ± SEM or as median [interquartile range (IQR)] if the distribution

242 was not normal. Baseline differences in laboratory and anthropometric variables were

243 assessed using a *t*-test and a Mann-Whitney U test for normally- and non-normally

244 distributed variables, respectively. Since most variables were found to be skewed, partial

245 Spearman's rank correlation was used to test the relationship between clinical variables

246 and *APOA1BP*, *SREBF* and *NOTCH* mRNAs adjusting for age, sex, BMI and country.

247   Analyses were performed with SPSS software (version 23, IBM SPSS Statistics) and

248   MATLAB R2014a. Levels of statistical significance were set at $P < 0.05$.

249   *Multivariate statistics.* Transcriptomic and metabolomic data multivariate statistics were

250   performed in MATLAB R2014a using in-house scripts. This included principal

251   component analysis (PCA) and orthogonal partial least squares (O-PLS) regression on

252   the mean-centered and unit variance-scaled variables. Unsupervised PCA was first

253   applied to visualize the global variance of the data sets to reveal intrinsic similarities,

254   possible confounders, and to identify strong and moderate outliers based on Hotelling's

255   T^2 and distance to the model, respectively. Then, supervised O-PLS regression models

256   were built to identify transcriptomic or metabolic features associated with the variables

257   of interest. Here, the omics profiles were used as the descriptor matrix (X) to predict the

258   response variable (Y). Significant features were selected based on the O-PLS regression

259   loadings adjusted for multiple testing using the Benjamini-Hochberg procedure for false

260   discovery rate (FDR). A pFDR < 0.05 was used as the reference feature selection

261   criterion. Finally, each individual variable identified form multivariate models was

262   further validated by partial Spearman's correlation adjusting for age, BMI, sex and

263   country. The predictive performance of the model (Q^2Y) was calculated using a leave-

264   one-out cross-validation approach and model validity was established by permutation

265   testing (1000 permutations).

266

267

Results

The baseline characteristics of the study participants are shown in Table 1.

ASSOCIATIONS OF *APOA1BP-SREBF-NOTCH* AXIS WITH CARDIOVASCULAR AND HAEMATOLOGICAL PARAMETERS

APOA1BP had significant inverse associations with several measures of cIMT, including RICAe, RproCCAi, LpreCCAi, LpreCCAe, mLCCA, mCCA, and mCA thickness (Figure 1a). Conversely, the expression levels of *SREBF1* correlated positively with cIMT measures in all segments of the left carotid artery (proximal, pre-bifurcation, internal) and plaque presence. *SREBF2* expression only correlated positively with RpreCCAe. Among the Notch receptors isoforms, *NOTCH2NL* had the strongest correlations with cIMT measures. In particular, it had strong correlations with all measures of the right internal carotid artery, whereas the expression levels of *NOTCH1* and *NOTCH4* were positively correlated with measures in the left carotid artery. Among these genes, only *SREBF1* had a positive association with the steatosis degree (Figure 1b). We also correlated the mean of the 12 CCA segments (mCCA) with the expression of all genes involved in cholesterol metabolism. Notably, *SREBF1* was the only gene that correlated positively with mCCA, whereas *APOA1BP* was amongst those having a stronger negative correlation (Figure 1c). Similar results were obtained when mCA was dichotomized based on a subclinical atherosclerosis cut-off >0.78 mm (Supplementary Figure 1a).

Given that atherosclerosis is considered a chronic inflammation disease, we examined the associations of atherosclerosis measures with the plasma levels of *N*-acetylglycoproteins (NAG) measured by NMR. NAG is a novel composite biomarker of systemic inflammation that integrates both protein levels and glycosylation states of several of the

13

292   most abundant acute phase proteins in serum [22]. As expected, NAG levels correlated

293   positively several cIMT measures (Supplementary Figure 1b). As, atherosclerosis is

294   driven by the progressive accumulation of lipids and leukocytes in the arterial wall, we

295   also analysed the association between the expression of the previous genes and lipid and

296   haematological parameters (Figure 1 and Supplementary Figure 1c). Specifically, the

297   expression of *APOA1BP* correlated negatively with WBC counts (Figure 1d), whereas

298   *SREBF1* and *NOTCH2NL* had a positive correlation with RBC (Figure 1e) and WBC

299   (Figure 1f) counts, respectively. The levels of HDL cholesterol (HDL-C) correlated

300   positively with *APOA1BP* expression (Figure 1g), but negatively with the expression of

301   *SREBF1* (Figure 1h). The later also had a positive correlation with the circulating

302   triglyceride (TG) concentration (Figure 1i). Total WBC counts, and in particular

303   lymphocytes and monocytes, also correlated positively with several atherosclerosis

304   measures (Supplementary Figure 1d).

305   *APOA1BP* **and** *SREBF* **ASSOCIATIONS WITH CHOLESTEROL AND NOTCH**

306   **PATHWAY GENES**

307   O-PLS models were built to identify genes involved in the Notch signalling (*n*=79 genes;

308   Supplementary Table 1) and cholesterol synthesis pathways (*n*=109 genes;

309   Supplementary Table 2) associated with *APOA1BP, SREBF1*, and *SREBF2* expression.

310   In the case of *APOA1BP*, significant O-PLS models with a good predictability were

311   obtained for both Notch pathway- (Figure 1j) and cholesterol pathway-associated genes

312   (Figure 1k). Significant genes identified from multivariate O-PLS regression models were

313   further validated by partial Spearman's correlation adjusting for age, BMI, sex, and

314   country (Figure 1l,m). Remarkably, the expression of all Notch receptors (*NOTCH1,*

315   *NOTCH2, NOTCH2NL, NOTCH3, NOTCH4*) and Jagged ligands (*JAG1, JAG2*), but not

316   that of delta-like ligands, was negatively associated with the *APOA1BP* expression

(Figure 1l). The expression of *APOA1BP* was also associated with the expression of cholesterol transporters such as *ABCA1* and *SCARB1* (Figure 1m). Significant O-PLS models were also obtained for the associations between *SREBF1* expression and genes from both Notch and cholesterol pathways (Supplementary Figure 2a,b). Notably, after partial Spearman's validation (Supplementary Figure 2c,d), the *SREBF1* expression was positively associated with *NOTCH1* and *ABCA1*, which we had found to be negatively associated with *APOAB1P*. Contrary to *SREBF1* results, we did not obtain a significant O-PLS between the expression of *SREBF2* and Notch pathway genes (Q^2Y=-0.21). Finally, an O-PLS regression model between cholesterol synthesis pathway genes and *NOTCH2NL* identified a negative association with *APOA1BP*, whereas *ABCA1* had the strongest positive association (Supplementary Figure 2e,f).

ASSOCIATIONS OF THE *APOA1BP-SREBF-NOTCH* AXIS WITH THE SERUM METABOLIC PROFILES

O-PLS regression models were built to identify serum metabolites associated with the expression of *APOA1BP, SREBF1,* and *SREBF2*. A significant O-PLS model was obtained for the prediction of *APOA1BP* expression levels from the serum metabolic profile (Figure 2a). Significant identified metabolites (Figure 2b) were further validated by partial Spearman's correlation (Figure 2c). We identified asparagine, glycerol, histidine, glycine, choline and citrate as positively associated with the expression of *APOA1BP*, whereas glyceryl of lipids and very low level cholesterol in VLDL were associated negatively. Metabolites associated positively with *APOA1BP* had negative associations with atherosclerosis measures, particularly LmCCA (Figure 2g-i). We also found a borderline significant negative ($r = -0.22$, $P = 0.051$) association between the expression of *APOA1BP* and the inflammatory marker NAG. Metabolome-wide association studies (MWAS) were also performed for the liver expression of *SREBF1* and

342   *SREBF2* using O-PLS multivariate regressions (Figure 2d,e and Supplementary Figure

343   3a) confirmed by partial Spearman's correlation (Figure 2 f and Supplementary Figure

344   3b). As expected, the expression of both genes was positively associated with several

345   lipids. They also had positive associations with inflammatory markers (NAG), branched-

346   chain amino acids (BCAA) (valine, isoleucine) and related catabolites (α-

347   ketoisovalerate), lactate and proline. Most of these metabolites had positive correlations

348   with cIMT measures. In particular, BCAA had positive associations with RICAe (Figure

349   2j-l), whereas NAG and α-ketoisovalerate had positive associations with mCA (R=0.27,

350   P=0.028; and R=0.33, P=0.006, respectively).

351

352   **ASSOCIATIONS OF HAEMATOPOIESIS AND LIPIDS WITH THE SERUM**

353   **METABOLIC PROFILES**

354   O-PLS regression models were also constructed to reveal associations between those

355   haematological and lipid parameters associated with the *APOA1BP-SREBF-NOTCH* axis

356   and the serum metabolome. A significant model with a strong predictive ability (Figure

357   3a) was obtained between the serum metabolic profile and the HDL-C levels. After

358   validation of the multivariate-identified metabolites by partial Spearman correlation,

359   metabolites associated with HDL-C were similar to those linked to *APOA1BP* and

360   *SREBF1* (Figure 3b). Hence, phosphocholine, choline, glycerol, and glycine, which

361   correlated positively with the expression of *APOA1BP*, also had a positive correlation

362   with HDL-C. Conversely, several lipids, inflammation-related metabolites (NAG),

363   BCAA (valine, isoleucine) and lactate, which were positively associated with *SREBF1*,

364   had a negative association with the circulating levels of HDL-C. Notably, the expression

365   of *NOTCH2NL* correlated negatively with the serum choline and phosphocholine levels

366 (Figure 3c,d) but positively with the BCAAs leucine and isoleucine (Figure 3e,f). It also

367 had a trend towards a positive association with NAG ($r = 0.20$, $P = 0.07$). A significant

368 O-PLS regression model was also obtained between the fasting serum TG levels and the

369 serum metabolome (Figure 3g). Patients with higher TG concentrations had higher levels

370 of several lipids, BCAAs and catabolites (isoleucine, α-ketoisovalerate), inflammation-

371 related metabolites (NAG), aspartate, proline, and acetone (Figure 3h). As expected, these

372 results agree with those metabolites positively associated with *SREBF1*. Interestingly,

373 several metabolites negatively associated with TG, including glycine, asparagine,

374 phosphocholine, glycerol, and histidine, were found to be positively associated with the

375 expression of *APOA1BP*. Finally, O-PLS regression models (Figure 3i) confirmed by

376 partial Spearman's correlation (Figure 3j) revealed that serum metabolites positively

377 associated with the leukocyte counts were similar to those associated with the expression

378 of *SREBF1*.

379

Discussion

381 Atherosclerosis and its progression is caused by lipid accumulation and local

382 inflammation of blood vessels, and is the major underlying cause of CVD. High plasma

383 concentrations of HDL have shown anti-atherogenic potential because HDL carries

384 excess cholesterol away from cells. Importantly, human studies have shown that HDL

385 cholesterol efflux capacity, a metric of HDL function that characterizes a key step in RCT,

386 may be athero-protective [1,3]. Recent *in vitro* and animal studies have shown that AIBP

387 may protect against atherosclerosis [5–8]. Here, we provided evidence for the first time,

388 to our knowledge, in humans of an inverse association between the expression of

389 *APOA1BP* and atherosclerosis measures.

390   *In vitro* studies have demonstrated a role of AIBP in promoting cholesterol efflux from

391   human umbilical vein endothelial cells to HDL and THP-1-derived macrophages, thereby

392   reducing lipid accumulation [5,6]. Mechanistically, AIBP enhances cholesterol efflux and

393   RCT by preventing ABCA1 protein degradation through facilitating its binding to

394   APOA1 on the cell membrane, thereby increasing ABCA1 levels [5]. Growing evidence

395   suggests that ABCA1 protects from atherosclerosis by exporting excess cholesterol from

396   cells to poorly lipidated APOA1, which is essential for the biogenesis of nascent HDL in

397   hepatocytes [23]. Recent animals studies have also highlighted the protection of AIBP

398   against atherosclerosis [7,8]. Hence, *Apoe*$^{-/-}$ mice with established atherosclerosis and

399   treated with recombinant adeno-associated virus (rAAV) to overexpress AIBP showed

400   reduction of atherosclerotic plaque size and inflammation but increased circulating HDL

401   levels and RCT to the liver [8]. In the latter model, AIBP was overexpressed in the aorta

402   and peritoneal macrophages, but mainly in the liver. The striking increase in ABCA1

403   protein levels in the aortas and peritoneal macrophages of AIBP-treated mice suggests

404   again that the effects of AIBP are mediated through ABCA1. In another animal study,

405   *Apoa1bp*$^{-/-}$*Ldlr*$^{-/-}$ mice fed a high-fat, high-cholesterol diet had exacerbated weight gain,

406   liver steatosis, hyperlipidaemia and atherosclerosis compared to *Ldlr*$^{-/-}$ mice [7]. In

407   addition, AAV-mediated overexpression of AIBP in *Ldlr*$^{-/-}$ mice protected against weight

408   gain, plasma lipid increase and atherosclerosis compared to controls.

409   In agreement with these results, we found negative associations between *APOA1BP*

410   expression levels and measures of cIMT. Conversely, we found a negative association

411   between the expression levels of *APOA1BP* and *ABCA1*. It is possible that in patients

412   with reduced hepatic *APOA1BP* expression, there is a compensatory response in the

413   expression of *ABCA1* to increase cholesterol efflux in the liver. In addition, although

414   treatment with AIBP increased ABCA1 protein levels in macrophages and *Apoe*$^{-/-}$ mice,

415   it did not alter the ABCA1 mRNA expression [5,8]. In fact, ABCA1 protein levels and

416   mRNA expression are usually discordant as ABCA1 protein levels are regulated by a

417   diverse posttranscriptional mechanism [24]. In contrast to ABCA1, which is important

418   for the generation of nascent HDL, scavenger receptor class B member 1 (SR-BI) is a

419   receptor for mature HDL and mediates selective uptake of HDL cholesteryl esters into

420   the liver as the final step in RCT [25]. Consistently, we found a significant association

421   between *APOA1BP* and *SCARB1,* which encodes the SR-BI protein, which was opposite

422   to the *APOA1BP-ABCA1* correlation.

423   In addition to the ABCA1 transporter pathway, the VLDL-APOB secretion pathway has

424   been proposed as the major pathway for the secretion of cholesterol from hepatocytes

425   together with TG into plasma [26]. Interestingly, the VLDL secretion pathway is

426   modulated by SREBP1c [27], encoded by *SREBF1*, which we found strongly positively

427   associated with atherosclerosis. Specifically, *SREBF1* correlated with cIMT measures in

428   all segments of the left carotid artery, which is in agreement the higher vulnerability of

429   the left carotid artery to atherosclerosis [28,29]. Hepatic expression of SREBP1c is also

430   increased in hepatic steatosis [30], which is consistent with our results. A recent study in

431   zebrafish has shown that Aibp2-mediated HPSC expansion through the up-regulation of

432   *Srebf2* (but not *Srebf1*), which predominantly regulates cholesterol synthesis [14].

433   Although we mainly found *SREBF1* associated with cIMT rather than *SREBF2*, it is worth

434   noting that *SREBF1* is transcribed into two variants: SREBP-1c, which solely regulates

435   lipid synthesis, and SREBP-1a, which controls both cholesterol and lipid synthesis. AIBP

436   also seem to modulate Notch signalling in mice and zebrafish [13,14]. In agreement with

437   these results, we found strong associations between the expression of *APOA1BP* and the

438   expression of all Notch signalling receptors and jagged ligands, but not with delta-like

439   ligands. Interestingly, jagged and delta-like ligands have shown opposite effects on

19

440   angiogenesis and regulation of T cells [31,32]. Consistent with a higher susceptibility of

441   the left carotid artery, we also found that *NOTCH1* and *NOTCH4* were positively

442   associated with left cIMT measures. Despite these associations, it is particularly

443   noticeable that the expression of human-specific gene *NOTCH2NL* had the strongest

444   associations with atherosclerotic measures. Interestingly, among all genes involved in

445   cholesterol metabolism, *NOTCH2NL* had the strongest positive association with *ABCA1*,

446   suggesting again a potential upregulation of *ABCA1* expression to increase cholesterol

447   efflux in patients with cIMT. Little is known about *NOTCH2NL*, but it has recently stood

448   out for its ability to enhance Notch signalling and expand human cortical progenitor cells

449   [33,34]. Consistent with this link between cholesterol metabolism and haematopoiesis,

450   we found significant associations among the expression of *APOA1BP, SREBF1* and

451   *NOTCH2NL,* and haematology and lipid parameters.

452   We found that the expression of transcripts *APOA1BP, SREBF1, SREBF2* and

453   *NOTCH2NL* was associated with several plasma metabolites. *APOA1BP* had positive

454   correlations with three amino acids (asparagine, histidine, glycine), glycerol, choline and

455   citrate. These amino acids were also associated positively with circulating HDL-C levels,

456   but negatively with TG and WBC counts. Glycine, histidine and asparagine were also

457   negatively associated with cIMT measures. Of note, among 35 plasma metabolites

458   quantified in 1049 individuals without coronary artery disease (CAD) and diabetes,

459   histidine was most strongly associated with lower risk for incident CAD, followed by

460   asparagine [35]. Low circulating levels of glycine have also been causally associated with

461   higher incidence of coronary heart disease [36].

462   *APOA1BP* expression also had a positive association with choline. Remarkably, both

463   choline and phosphocholine levels had very strong positive associations with HDL-C but

464   correlated negatively with the expression of *NOTCH2NL*. There is evidence suggesting a

connection between choline and HDL metabolism. Hence, phosphatidylcholine is the major phospholipid component of all plasma lipoproteins and the biosynthesis of phosphatidylcholine in the liver is critical for the synthesis and secretion of HDL and VLDL [37]. Interestingly, *APOA1BP* correlated negatively with the expression of liver enzymes involved in the synthesis of phosphatidylcholine from choline and phosphocholine, suggesting an up-regulation of the synthesis of phosphatidylcholine in patients with lower *APOA1BP* to increase assembly and clearance of lipoproteins. Due to the role of lecithin–cholesterol acyltransferase (LCAT) in the formation of HDL and RCT, we also assessed the relation between the expression levels of *APOA1BP* and *LCAT* and found a positive association (R=0.27, P=0.01). Interestingly, although studies in the general population gave inconsistent results, patients with LCAT deficiency have shown a significant increase in the incidence of CVD [38]. Despite the associations with *APOA1BP* and HDL, neither choline nor phosphocholine had associations with measures of atherosclerosis. Previous studies have shown inconsistent associations between circulating choline and phosphocholine levels and cardiovascular events. Both metabolites were positively associated with the risk of CVD and stroke after 4.8 years of follow-up in participants at high cardiovascular risk within the PREDIMED study, but no associations were found between 1-year changes in these metabolites and the incidence of CVD [39]. Similarly, higher plasma choline levels were associated with increased risk of major cardiac events after 3 years of follow-up in patients undergoing elective diagnostic coronary angiography, but they did not predict risk when trimethylamine *N*-oxide, a choline metabolite, was added to the model [40]. In cross-section studies higher plasma choline concentrations have been associated with an unfavourable cardiometabolic risk factor profile, including lower HDL levels [41,42]. However, it is worth noting that plasma choline levels do not reflect choline status because disturbances

490   in circulating choline arise early in disease development [43,44] and circulating levels

491   may depend on lipid overload. In this sense, plasma choline metabolites differed between

492   normal and overweight men [45]. In addition, both low and high plasma concentrations

493   may be associated with adverse effects [46].

494   As expected, the expression levels of *SREBF1* and *SREBF2* had positive associations with

495   several lipids. These genes also correlated positively with circulating levels of BCAA and

496   catabolites, NAG and lactate. Except for lactate, we found that these metabolites were all

497   positively associated with measure of cIMT. Several cross-section and prospective cohort

498   studies revealed positive associations of BCAA with major metabolic disorders [47] and

499   recently they have also been positively associated with risk factors of CAD, in particular

500   cIMT [48]. Interestingly, we also found a positive correlation between the expression

501   levels of *NOTCH2NL* and BCAA. In line with atherosclerosis being a chronic

502   inflammatory disorder, we found a consistent association between NAG, a novel marker

503   of chronic inflammation [22], and cIMT measures. Consistently, we found that NAG

504   levels were the strongest predictors of WBC counts, which also had positive associations

505   with BCAA. Supporting the role of the *APOA1BP-SREBF-NOTCH* axis and

506   inflammation in atherosclerosis, we also found that NAG levels were negatively

507   associated with the expression of *APOA1BP*, but positively with that of *SREBF1,*

508   *SREBF2,* and *NOTCH2NL*. Finally, most of the metabolites associated with *SREBF1* and

509   *SREBF2* had similar associations with TG, but opposite to those of HDL.

510   The main limitation of our study is its cross-sectional nature. Therefore, we cannot infer

511   cause-effect relationships. Second, the results cannot be extrapolated to the general

512   population, as subjects in the current study were morbidly obese. In addition, we did not

513   measure AIBP protein levels. The strengths of the present work include the use of two

514   independent well-characterized cohorts, which allowed the control for potential

515 confounders, with comprehensive metabolomic, transcriptomic, liver biopsy and cIMT

516 data.

517 In conclusion, our findings demonstrate, for the first time, an atheroprotective association

518 between the expression levels of *APOA1BP* and atherosclerosis in humans. We have also

519 shown a connection between *APO1ABP,* Notch signalling and inflammation with

520 atherosclerosis.

521

522 **Acknowledgments**

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690

Figure Legends

692 **Figure 1. Associations of expression levels of *APOA1BP, SREBFs,* and *NOTCHs* gens**

693 **with cIMT, steatosis, haematology and lipid measures and cholesterol and notch**

694 **pathway genes. a)** Heatmap displaying the partial Spearman's correlation coefficients

695 (adjusted for age, BMI, sex, and cohort) between genes and cIMT measures. *$*p<0.05$,

696 *$**p<0.01$, *$***p<0.001$. **b)** Association of expression levels of *SREBF1* with the steatosis

697 degree (ANCOVA and Tukey-kramer tests). **c)** Cholesterol metabolism pathway genes

698 significantly associated with mean CCA based on partial Spearman's correlation adjusted

699 for age, BMI, sex, and cohort. **d-i)** Scatter plots between expression levels of selected

700 genes and haematological and lipid parameters. Partial Spearman's correlation and

701 significance values adjusted for age, BMI, sex, and cohort are shown. **j-k)** Permutation

702 test for the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-

703 PLS models between the expression levels of *APOA1BP* and the expression levels of

704 genes involved in the Notch signalling and cholesterol pathways, respectively. **l-m)**

705 Significant Notch and cholesterol pathway genes associated with *APOA1BP* after further

706 validation of the O-PLS identified genes by partial Spearman's correlation adjusting for

707 age, sex, BMI, and country.

708 **Figure 2. Associations of metabolomics data with the expression levels of *APOA1BP,***

709 ***SREBF1,*and cIMT measures. a)** Permutation test for the goodness-of-fit (R^2Y) and

710 goodness of prediction (Q^2Y) obtained from the O-PLS models between the expression

711 levels of *APOA1BP* and the serum metabolome. **b)** Significant serum metabolites

712 obtained from the O-PLS model. Statistically significant metabolites are coloured in red

713 if positively associated with *APOA1BP* and blue if negatively associated. **c)** Metabolites

714 associated with *APOA1BP* after further validation of the O-PLS identified metabolites by

715 partial Spearman's correlation adjusting for age, sex, BMI, and country. **d)** Permutation

716   test for the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-

717   PLS models between the expression levels of *SREBF1* and the serum metabolome. **e)**

718   Significant serum metabolites obtained from the O-PLS model. Statistically significant

719   metabolites are coloured in red if positively associated with *SREBF1* and blue if

720   negatively associated. **f)** Metabolites associated with *SREBF1* after further validation of

721   the O-PLS identified metabolites by partial Spearman's correlation adjusting for age, sex,

722   BMI, and country. **g-l)** Scatter plots between selected metabolites and cIMT measures.

723   Partial Spearman's correlation and significance values adjusted for age, BMI, sex, and

724   cohort are shown.

725   **Figure 3. Associations of metabolomics data with the lipid parameters, WBC, and**

726   ***NOTCH2NL* expression levels. a)** Permutation test for the goodness-of-fit (R^2Y) and

727   goodness of prediction (Q^2Y) obtained from the O-PLS models between the expression

728   levels of HDL-C and the serum metabolome. **b)** Metabolites associated with HDL-C after

729   further validation of the O-PLS identified metabolites by partial Spearman's correlation

730   adjusting for age, sex, BMI, and country. **c-f)** Scatter plots between selected metabolites

731   and *NOTCH2NL* expression levels. Partial Spearman's correlation and significance

732   values adjusted for age, BMI, sex, and cohort are shown. **g)** Permutation test for the

733   goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-PLS models

734   between the expression levels of TG and the serum metabolome. **h)** Metabolites

735   associated with TG after further validation of the O-PLS identified metabolites by partial

736   Spearman's correlation adjusting for age, sex, BMI, and country. **i)** Permutation test for

737   the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-PLS

738   models between the expression levels of WBC and the serum metabolome. **j)** Metabolites

739   associated with WBC after further validation of the O-PLS identified metabolites by

740   partial Spearman's correlation adjusting for age, sex, BMI, and country.

741 **Supplementary Figure 1. a)** Cholesterol metabolism pathway genes significantly

742 associated with mean CA based on partial Spearman's correlation adjusted for age, BMI,

743 sex, and cohort. **b)** Heatmap displaying the partial Spearman's correlations (adjusted for

744 age, BMI, sex, and cohort) between cIMT measures and inflammation measured by NMR

745 plasma levels of *N*-acetylglycoproteins (NAG). **c)** Heatmap displaying the partial

746 Spearman's correlation coefficients (adjusted for age, BMI, sex, and cohort) between

747 genes and haematological parameters. **d)** Heatmap displaying the partial Spearman's

748 correlation coefficients (adjusted for age, BMI, sex, and cohort) between haematological

749 parameters and cIMT measures.

750 **Supplementary Figure 2. a,b)** Permutation test for the goodness-of-fit (R^2Y) and

751 goodness of prediction (Q^2Y) obtained from the O-PLS models between the expression

752 levels of *SREBF1* and the expression levels of genes involved in Notch signalling and

753 cholesterol synthesis pathways, respectively. **c,d)** Significant Notch signalling and

754 cholesterol pathway genes associated with *SREBF1* after further validation of the O-PLS

755 identified genes by partial Spearman's correlation adjusting for age, sex, BMI, and

756 country, respectively. **e)** Permutation test for the goodness-of-fit (R^2Y) and goodness of

757 prediction (Q^2Y) obtained from the O-PLS models between the expression levels of

758 *NOTCH2NL* and the expression levels of genes involved in cholesterol synthesis

759 pathway. **f)** Significant cholesterol pathway genes associated with *NOTCH2NL* after

760 further validation of the O-PLS identified genes by partial Spearman's correlation

761 adjusting for age, sex, BMI, and country.

762 **Supplementary Figure 3. a)** Permutation test for the goodness-of-fit (R^2Y) and goodness

763 of prediction (Q^2Y) obtained from the O-PLS models between the expression levels of

764 *SREBF2* and the serum metabolome. **b)** Significant serum metabolites obtained from the

765 O-PLS model. Statistically significant metabolites are coloured in red if positively

766   associated with *SREBF2* and blue if negatively associated. **c)** Metabolites associated with

767   *SREBF2* after further validation of the O-PLS identified metabolites by partial

768   Spearman's correlation adjusting for age, sex, BMI, and country

769

Table 1. Baseline characteristics of participants.

Variables	All (*n*=78)
Age (years)	42.4 ± 1.16
BMI (kg/m^2)	46.2 (42.4-51.1)
Waist circumference (cm)	127.0 (119.5-138.0)
Sex (women, %)	79.2
SBP (mmHg)	134.0 (122.5-144.0)
DBP (mmHg)	81.0 (75.0-90.0)
Biochemistry	
Glucose (mg/dL)	95.0 (88.5-101.5)
HOMA-IR	4.50 (2.95-6.90)
Triglycerides (mg/dL)	112.0 (82.0-143.5)
Total cholesterol (mg/dL)	197.5 ± 4.6
LDL cholesterol (mg/dL)	131.0 ± 4.0
HDL cholesterol (mg/dL)	45.0 (40.0-51.2)
Haematology	
Eosinophils	150.0 (100.0-200.0)
Neutrophils	4810 (3665-5795)
Lymphocytes	2360 ± 86.9
Monocytes	500 (420-600)
Total WBC	7350 (6385-9030)
RBC	4.66 (4.46-4.91)
Liver	
HsCRP	0.88 (0.47-1.39)
AST	19.0 (15.0-28.0)
ALT	30.0 (22.0-43.5)
Steatosis grade (%):	
Grade 0	15.6
Grade 1	33.8
Grade 2	24.7
Grade 3	26.0
Atherosclerosis	
RproCCAi	0.70 (0.60-0.80)
RproCCAe	0.70 (0.60-0.80)
RpreCCAi	0.79 ± 0.03
RpreCCAe	0.72 (0.60-0.80)
RICAi	0.70 (0.60-0.90)
RICAe	0.70 (0.54-0.85)
mRCCA	0.73 ± 0.02
mRICA	0.71 ± 0.02
LproCCAi	0.70 (0.60-0.85)
LproCCAe	0.69 (0.60-0.80)
LpreCCAi	0.80 (0.70-1.0)
LpreCCAe	0.84 ± 0.03
LICAi	0.77 (0.57-0.90)
LICAe	0.70 (0.60-0.87)
mLCCA	0.76 ± 0.02
mLICA	0.73 ± 0.03
mCCA	0.74 ± 0.02
mCA	0.73 ± 0.02

771 Values are expressed as means ± SEM for normally distributed variables and median [IQR] for non-normally
772 distributed variables. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HsCRP, high sensitivity

773 C-reactive protein; e, external; i, internal; ICA, internal carotid artery; L, left; m, mean; preCCA, pre bifurcation
774 common carotid artery; proCCA, proximal segment common carotid artery; R, right; RBC, red blood cells; WBC,
775 white blood cells.

The *APOA1BP-SREBF-NOTCH* axis is associated with reduced atherosclerosis risk in morbidly obese patients

Jordi Mayneris-Perxachs[1,2], Josep Puig[1], Rémy Burcelin[3,4], Marc-Emmanuel Dumas[5,6], Richard H. Barton[5], Lesley Hoyles[7], Massimo Federici[8], José-Manuel Fernández-Real[1,2*]

[1] Department of Endocrinology, Diabetes and Nutrition, Hospital of Girona "Dr Josep Trueta", Departament de Ciències Mèdiques, University of Girona, Girona Biomedical Research Institute (IdibGi), Girona, Spain

[2] CIBERobn Pathophysiology of Obesity and Nutrition, Instituto de Salud Carlos III, Madrid, Spain

[3] Institut National de la Santé et de la Recherche Médicale (INSERM), Toulouse, France

[4] Université Paul Sabatier (UPS), Unité Mixte de Recherche (UMR) 1048, Institut des Maladies Métaboliques et Cardiovasculaires (I2MC), Team 2: 'Intestinal Risk Factors, Diabetes, Dyslipidemia, and Heart Failure' F-31432 Toulouse Cedex 4, France

[5] Section of Computational and Systems Medicine, Division of Integrative Systems Medicine, Department of Metabolism, Digestion and Reproduction, Imperial College London, Exhibition Road, London SW7 2AZ, United Kingdom

[6] Section of Genomic and Environmental Medicine, National Heart & Lung Institute, Imperial College London, Dovehouse Street, SW3 6KY London, United Kingdom

[7] Department of Biosciences, Nottingham Trent University, Clifton Campus, Nottingham NG11 8NS, United Kingdom

[8] Department of Systems Medicine, University of Rome Tor Vergata, Via Montpellier 1 00133, Rome, Italy

Corresponding author:

José-Manuel Fernández-Real, M.D., PhD.

Department of Diabetes, Endocrinology and Nutrition.

Dr. Josep Trueta University Hospital. Girona Biomedical Research Institute (IdIBGi).

Carretera de França s/n, 17007, Girona, Spain.

Phone: +34 972940200 / Fax: +34 97294027.

E-mail: jmfreal@idibgi.org

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40

Abbreviations

AAV, adeno-associated virus; ABCA1, ATP-binding cassette transporter A1; AIBP, apolipoprotein A-I binding protein; APOA1, apolipoprotein A1; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCAA, branched-chain amino acids; CCA; common carotid arteries; cIMT, carotid intima-media thickness; CAD, coronary artery disease; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HPSC, hematopoietic progenitor and stem cells; HsCRP, high sensitive C-reactive protein; ICA, internal carotid arteries; LCAT, lecithin-cholesterol acyltransferase; MWAS, metabolite-wide association studies; NAG, N-acetylglycoproteins; O-PLS, orthogonal partial least squares; PCA, principal component analysis; preCCA, pre bifurcation common carotid arteries; proCCA, proximal segment common carotid arteries; RBC, red blood cells; RCT, reverse cholesterol transport; SR-BI; scavenger receptor class B member 1; TG, triglycerides; WBC, white blood cells

Abstract

Background & Aims: Atherosclerosis is characterized by an inflammatory disease linked to excessive lipid accumulation in the artery wall. The Notch signalling pathway has been shown to play a key regulatory role in the regulation of inflammation. Recently, *in vitro* and pre-clinical studies have shown that apolipoprotein A-I binding protein (AIBP) regulates cholesterol metabolism (SREBP) and NOTCH signalling (haematopoiesis) and may be protective against atherosclerosis, but the evidence in humans is scarce.

Methods: We evaluated the *APOA1BP-SREBF-NOTCH* axis in association with atherosclerosis in two well-characterized cohorts of morbidly obese patients ($n = 78$) within the FLORINASH study, including liver transcriptomics, ^{1}H-NMR plasma metabolomics, high-resolution ultrasonography evaluating carotid intima-media thickness (cIMT), and haematological parameters.

Results: The liver expression levels of *APOA1BP* were associated with lower cIMT and leukocyte counts, a better plasma lipid profile and higher circulating levels of metabolites associated with lower risk of atherosclerosis (glycine, histidine and asparagine). Conversely, liver *SREBF* and *NOTCH* mRNAs were positively associated with atherosclerosis, liver steatosis, an unfavourable lipid profile, higher leukocytes and increased levels of metabolites linked to inflammation and CVD such as branched-chain amino acids and glycoproteins. *APOA1BP* and NOTCH signalling also had a strong association, as revealed by the negative correlations among *APOA1BP* expression levels and those of all NOTCH receptors and jagged ligands.

3

77 **Conclusions:** We here provide the first evidence in human liver of the putative

78 *APOA1BP-SREBF-NOTCH* axis signalling pathway and its association with

79 atherosclerosis and inflammation.

80　Atherosclerosis is the major cause of cardiovascular disease (CVD), the leading cause of

81　death worldwide. Several studies have shown the anti-atherogenic potential of the high-

82　density lipoprotein (HDL)-mediated reverse cholesterol transport (RCT) [1]. In this

83　process, excess cholesterol is transported from macrophages and peripheral tissues back

84　to the liver for excretion. A critical part of RCT is cholesterol efflux, in which intracellular

85　cholesterol is released and collected by apolipoprotein A1 (APOA1), the major

86　component of HDL [2]. In fact, the cholesterol efflux potential of HDL is a better

87　predictor of CVD than circulating HDL levels [3]. The ATP-binding cassette transporter

88　A1 (ABCA1), a cell-membrane protein, is the major transporter that mediates this

89　assembly of cholesterol with APOA1, which is the rate-limiting step in the formation of

90　nascent HDLs.

91　The APOA1 binding protein (AIBP), encoded by *APOA1BP* in humans, is a secreted

92　protein physically associated with APOA1 [4] that enhances cholesterol efflux *in vitro*

93　from macrophages [5] and endothelial cells [6] in the presence of APOA1 or HDL, partly

94　by facilitating apoA1 binding to ABCA1 and preventing ABCA1 degradation [5].

95　Recently, AIBP has shown to protect against atherosclerosis *in vivo* in *Apoe*^{-/-} and *Ldlr*^{-/-}

96　mice by promoting cholesterol efflux and ameliorating inflammation [7,8]. Human

97　*APOA1BP* mRNA is ubiquitously expressed and abundant in most human secretory

98　organs, with the highest expression in kidney, heart, liver, thyroid gland, adrenal gland,

99　and testis [4]. It is of note that kidney and liver are the major sites of APOA1 catabolism.

100　Atherosclerosis is characterized by progressive accumulation of lipids and leukocytes in

101　the intima layer of the arterial wall. It is considered a chronic inflammation disease with

102　monocytes/macrophages, the main immune cells of the innate immune response, playing

103　a major role in all stages of atherosclerosis [9]. Notably, the Notch pathway is well-

104　recognized as a major regulator of cell fate in stem cells and the differentiation of the

105 various cell types of the immune system [10]. Recently, the Notch signalling pathway

106 was shown to play an important role in the onset and progression of atherosclerosis by

107 promoting inflammation through induction of a pro-inflammatory M1 phenotype in

108 macrophages, a switch towards a Th-1 like inflammatory phenotype in differentiated

109 regulatory T cells, and promoting CD8 cytotoxic T cells [11].

110 Recent evidence suggests a control of hematopoietic progenitor and stem cells (HPSC)

111 by cholesterol pathways [12], thereby linking haematopoiesis with atherosclerosis.

112 Interestingly, a connection between AIBP-mediated cholesterol metabolism and Notch

113 signalling has been described recently. AIBP was shown to regulate Notch signalling

114 through relocalization of γ-secretase from lipid to non-lipid rafts in mice [13] and

115 zebrafish Aibp2-mediated cholesterol efflux was shown to active endothelial Srebp2,

116 which in turn trans-activates Notch and promotes HSPC emergence. These observations

117 suggest a role for the AIBP-SREBP-NOTCH axis in atherosclerosis [14]. Although the

118 bone marrow is the primary site of haematopoiesis in adults, the liver is the main site

119 during prenatal development and hepatic haematopoiesis is believed to persist into

120 adulthood [15,16].

121 No study has, to date, addressed the potential association between AIBP and Notch

122 signalling in humans. Here, we studied this connection in two well-characterized cohorts

123 of morbidly obese patients recruited to the FLORINASH study (17) combining hepatic

124 transcriptome, plasma metabolome, and carotid intima-media thickness (cIMT)

125 measures.

126

6

127 **Subjects and Methods**

128 **STUDY POPULATION AND SAMPLE COLLECTION**

129 The study population ($n = 78$) included two cohorts of morbidly obese patients aged 22

130 to 61 years old recruited at the Endocrinology Service of the Hospital Universitari de

131 Girona Dr Josep Trueta (Girona, Spain, $n = 32$) and at the Center for Atherosclerosis of

132 Policlinico Tor Vergata University of Rome (Rome, Italy, $n = 46$), as part of the

133 FLORINASH study [17]. All subjects gave written informed consent, which was

134 validated and approved by the ethical committees of the Hospital Universitari Dr Josep

135 Trueta (Comitè d'Ètica d'Investigació Clínica, approval number 2009 046) and

136 Policlinico Tor Vergata University of Rome (Comitato Etico Indipendente, approval

137 number 28-05-2009). Inclusion criteria included Caucasian origin, stable body weight 3

138 months before the study, free of any infection one month preceding the study and absence

139 of any systemic disease. Exclusion criteria included presence of liver disease and thyroid

140 dysfunction, alcohol consumption (> 20 g/day), hepatitis B, drug-induced liver injury,

141 and dyslipidaemia medication. Plasma samples were obtained during the week before

142 elective gastric bypass surgery, during which a liver biopsy was sampled. All samples

143 were stored at −80 °C. Liver samples were collected in RNAlater, fragmented and

144 immediately flash frozen in liquid nitrogen before storage at −80 °C.

145

146 **ANTHROPOMETRIC AND LABORATORY MEASUREMENTS**

147 Blood pressure was measured using a blood pressure monitor (Hem-703C, Omron,

148 Barcelona, Spain), with the subject seated and after 5 min of rest. Three readings were

149 obtained and the mean value was used in the analyses. Waist circumference was measured

150 with a soft tape midway between the lowest rib and the iliac crest. Obesity was considered

151 as a body mass index (BMI) ≥ 30 kg/m^2.

152 After 8 h fasting, blood was drawn for the measurement of plasma lipids, glucose and

153 insulin. Glucose and lipids were determined by standard laboratory methods. Intra-assay

154 and inter-assay coefficients of variation (CV) were less than 4 %. Serum insulin was

155 measured in duplicate using a monoclonal immunoradiometric assay (Medgenix

156 Diagnostics, Fleunes, Belgium). The intra-assay CV was 5.2 % at a concentration of 10

157 mU/L and 3.4 % at 130 mU/L. The interassay CVs were 6.9 % and 4.5 % at 14 and 89

158 mU/L, respectively. Glycated haemoglobin (HbA1c) was measured by high-pressure

159 liquid chromatography using a fully automated glycated haemoglobin analyser system

160 (Hitachi L-9100; Hitachi, Tokyo, Japan). Insulin resistance was determined by the

161 homeostasis model assessment of insulin resistance (HOMA-IR). High sensitive C-

162 reactive protein (HsCRP) was determined by ultrasensitive assay (Beckman, Fullerton,

163 CA), with intra-and inter-assay CVs of 4 %. Serum alanine aminotransferase (ALT) and

164 aspartate aminotransferase (AST) levels were determined using enzymatic methods.

165

166 **LIVER HISTOLOGY**

167 Liver biopsies were analysed by a single pathologist expert in hepatic pathology. For each

168 liver sample, haematoxylin and eosin, reticulin, and Masson's trichrome staining were

169 performed. Hepatic steatosis grade was determined according to the scoring system for

170 NAFLD and defined as absent (grade 0: <5% steatosis), mild (grade 1: 5-33% steatosis),

171 moderate (grade 2: >33-66% steatosis) or severe (grade 3: >66% steatosis) [18]. Images

172 were independently evaluated by two radiologists blinded to clinical and laboratory data.

173

174

¹H NUCLEAR MAGNETIC RESONANCE (¹H-NMR) METABOLIC PROFILING

Metabolomics analyses have been previously described [17]. Briefly, plasma samples were thawed at room temperature and 350 μL aliquots were carefully placed in 5-mm NMR tubes. Then, 150 μL of saline solution (0.9 % NaCl) were added and the mixture was gently vortexed. Spectroscopic analyses were performed on a Bruker DRX600 spectrometer equipped with either a 5-mm TXI probe operating at 600.13 MHz or a 5-mm BBI probe operating at 600.44 MHz. Spectra were acquired using a water suppressed Carr-Purcell-Meiboom-Gill using the Bruker program *cpmgpr* (RD 90º-(τ-180º-τ) n-acquire). A RD of 2 s was employed for net magnetization relaxation, during which noise irradiation was applied in order to suppress the large water proton signal. A number of loops $n = 100$ and a spin-echo delay $\tau = 400$ μs was used to allow spectral editing through T2 relaxation and therefore attenuation of broad signals. For each sample, 128 scans were recorded in 32K data points with a spectral with of 20 ppm. All NMR spectra were processed using Topspin (Bruker Biospin, UK). Free induction decays were multiplied by an exponential function corresponding to a line broadening of 0.3 Hz and Fourier transformed. Spectra were automatically phased, baseline-corrected and referenced to the anomeric doublet of glucose (5.23 ppm). The spectra were all then imported to MATLAB and the region around the water resonance (δ=4.5-5.0) was removed.

TRANSCRIPTOMICS

Transcriptomic analyses have been previously described [17]. Briefly, RNA from liver biopsy samples was extracted using standard extraction protocols (TRIzol) by Miltenyi Biotec as previously reported. RNA quality (gel images, RNA integrity number and electropherograms) was assessed using an Agilent 2100 Bioanalyzer platform (Agilent

200 Technologies). An RNA integrity number >6 was considered sufficient for gene

201 expression experiments [19]. One-hundred ng of total RNA were used for linear T7-based

202 amplification of RNA for each sample. cDNA was prepared by amplification of the RNA

203 and labelled with Cy3 using the Agilent Low Input Quick Amp Labeling Kit according

204 to the manufacturer's instructions. The amounts of cDNA and dye that were incorporated

205 were measured by an ND-1000 spectrophotometer (NanoDrop Technologies).

206 Hybridization of the Agilent Whole Human Genome Oligo Microarrays $4 \times 44K$ was

207 done following the Agilent 60-mer oligo microarray processing protocol using the

208 Agilent Gene Expression Hybridization Kit. The fluorescence signals of the hybridized

209 Agilent microarrays were detected using Agilent's Microarray Scanner after washing

210 with Agilent Gene Expression Wash Buffer twice and with acetonitrile once. Feature

211 intensities were determined using Agilent Feature Extraction Software. Microarray data

212 were processed and normalized using R and the BioConductor package LIMMA (Linear

213 Models for Microarray Data) [20]. Raw data quality was assessed using pseudoMA and

214 box plots. A background correction was applied and normalization of the green channel

215 between arrays was done using 'cyclicloess' between pairs of arrays. Control and low-

216 expressed probes were removed and only those probes brighter than the negative controls

217 ($\geq$ 10%) on at least one array were kept. Batch-corrected data were obtained using

218 removeBatchEffect based on 'Batch' [20]. Probes with no associated gene ID were

219 removed. Finally, data were averaged based on an association to a particular gene.

220

221

HIGH-RESOLUTION ULTRASONOGRAPHY CAROTID EVALUATION

Carotid arteries were assessed using a Siemens Acuson S2000 (Mochida Siemens Medical System, Tokyo, Japan) ultrasound system with a 7.5 MHz linear array transducer. Images were independently evaluated by two radiologists blinded to clinical and laboratory data. cIMT values were manually measured in 12 carotid segments: internal (i) and external walls (e) of the right and left common carotid arteries (CCA) in a proximal segment (proCCA), in a plaque-free segment 10 mm from the bifurcation (preCCA) and in the internal carotid arteries (ICA). For the left and right arteries, the mean CCA and ICA values for each subject were calculated from these four measurements (mRCCA and mLCCA) and two measurement (mRICA and mLICA), respectively. The average of all measurements was reported as overall cIMT (mCA). Sub-clinical atherosclerosis was defined as a cut-off value of overall cIMT >0.78 mm [21]. A plaque was defined as a focal thickening ≥1.2 mm in any of 12 carotid segments (near and far walls of the right and left common carotid arteries, bifurcation and internal carotid artery).

STATISTICAL ANALYSES

Univariate statistics. Data distribution and normality of variables were checked visually, and using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Baseline characteristics are presented as means ± SEM or as median [interquartile range (IQR)] if the distribution was not normal. Baseline differences in laboratory and anthropometric variables were assessed using a *t*-test and a Mann-Whitney U test for normally- and non-normally distributed variables, respectively. Since most variables were found to be skewed, partial Spearman's rank correlation was used to test the relationship between clinical variables and *APOA1BP*, *SREBF* and *NOTCH* mRNAs adjusting for age, sex, BMI and country.

247   Analyses were performed with SPSS software (version 23, IBM SPSS Statistics) and

248   MATLAB R2014a. Levels of statistical significance were set at $P < 0.05$.

249   *Multivariate statistics.* Transcriptomic and metabolomic data multivariate statistics were

250   performed in MATLAB R2014a using in-house scripts. This included principal

251   component analysis (PCA) and orthogonal partial least squares (O-PLS) regression on

252   the mean-centered and unit variance-scaled variables. Unsupervised PCA was first

253   applied to visualize the global variance of the data sets to reveal intrinsic similarities,

254   possible confounders, and to identify strong and moderate outliers based on Hotelling's

255   T^2 and distance to the model, respectively. Then, supervised O-PLS regression models

256   were built to identify transcriptomic or metabolic features associated with the variables

257   of interest. Here, the omics profiles were used as the descriptor matrix (X) to predict the

258   response variable (Y). Significant features were selected based on the O-PLS regression

259   loadings adjusted for multiple testing using the Benjamini-Hochberg procedure for false

260   discovery rate (FDR). A pFDR < 0.05 was used as the reference feature selection

261   criterion. Finally, each individual variable identified form multivariate models was

262   further validated by partial Spearman's correlation adjusting for age, BMI, sex and

263   country. The predictive performance of the model (Q^2Y) was calculated using a leave-

264   one-out cross-validation approach and model validity was established by permutation

265   testing (1000 permutations).

266

267

Results

The baseline characteristics of the study participants are shown in Table 1.

ASSOCIATIONS OF *APOA1BP-SREBF-NOTCH* AXIS WITH CARDIOVASCULAR AND HAEMATOLOGICAL PARAMETERS

APOA1BP had significant inverse associations with several measures of cIMT, including RICAe, ~~RproCAAi~~RproCCAi, LpreCCAi, LpreCCAe, mLCCA, mCCA, and mCA thickness (Figure 1a). Conversely, the expression levels of *SREBF1* correlated positively with cIMT measures in all segments of the left carotid artery (proximal, pre-bifurcation, internal) and plaque presence. *SREBF2* expression only correlated positively with RpreCCAe. Among the Notch receptors isoforms, *NOTCH2NL* had the strongest correlations with cIMT measures. In particular, it had strong correlations with all measures of the right internal carotid artery, whereas the expression levels of *NOTCH1* and *NOTCH4* were positively correlated with measures in the left carotid artery. Among these genes, only *SREBF1* had a positive association with the steatosis degree (Figure 1b). We also correlated the mean of the 12 CCA segments (mCCA) with the expression of all genes involved in cholesterol metabolism. Notably, *SREBF1* was the only gene that correlated positively with mCCA, whereas *APOA1BP* was amongst those having a stronger negative correlation (Figure 1c). Similar results were obtained when mCA was dichotomized based on a subclinical atherosclerosis cut-off >0.78 mm (Supplementary Figure 1a).

Given that atherosclerosis is considered a chronic inflammation disease, we examined the associations of atherosclerosis measures with the plasma levels of *N*-acetylglycoproteins (NAG) measured by NMR. NAG is a novel composite biomarker of systemic inflammation that integrates both protein levels and glycosylation states of several of the

13

292 most abundant acute phase proteins in serum [22]. As expected, NAG levels correlated

293 positively several cIMT measures (Supplementary Figure 1b). As, Aatherosclerosis is

294 characterized driven by the progressive accumulation of lipids and leukocytes in the

295 arterial wall. . Therefore, we also analysed the association between the expression of the

296 previous genes and lipid and haematological parameters (Figure 1 and Supplementary

297 Figure 1c). Specifically, Tthe expression of *APOA1BP* correlated negatively with WBC

298 counts (Figure 1d), whereas *SREBF1* and *NOTCH2NL* had a positive correlation with

299 RBC (Figure 1e) and WBC (Figure 1f) counts, respectively. The levels of HDL

300 cholesterol (HDL-C) correlated positively with *APOA1BP* expression (Figure 1g), but

301 negatively with the expression of *SREBF1* (Figure 1h). The later also had a positive

302 correlation with the circulating triglyceride (TG) concentration (Figure 1i). Total WBC

303 counts, and in particular lymphocytes and monocytes, also correlated positively with

304 several atherosclerosis measures (Supplementary Figure 1d).

305 *APOA1BP* **and** *SREBF* **ASSOCIATIONS WITH CHOLESTEROL AND NOTCH**

306 **PATHWAY GENES**

307 O-PLS models were built to identify genes involved in the Notch signalling (*n*=79 genes;

308 Supplementary Table 1) and cholesterol synthesis pathways (*n*=109 genes;

309 Supplementary Table 2) associated with *APOA1BP*, *SREBF1*, and *SREBF2* expression.

310 In the case of *APOA1BP*, Ssignificant O-PLS models with a good predictability were

311 obtained for both Notch pathway- (Figure 1j) and cholesterol pathway-associated genes

312 (Figure 1k). Significant genes identified from multivariate O-PLS regression models were

313 further validated by partial Spearman's correlation adjusting for age, BMI, sex, and

314 country (Figure 1l,m). Remarkably, the expression of all Notch receptors (*NOTCH1,*

315 *NOTCH2, NOTCH2NL, NOTCH3, NOTCH4*) and Jagged ligands (*JAG1, JAG2*), but not

316 that of delta-like ligands, was negatively associated with the *APOA1BP* expression

14

317   (Figure 1l). The expression of *APOA1BP* was also associated with the expression of

318   cholesterol transporters such as *ABCA1* and *SCARB1* (Figure 1m). Significant O-PLS

319   models were also obtained for the associations between *SREBF1* expression and genes

320   from both Notch and cholesterol pathways (Supplementary Figure 2a,b). Notably, after

321   partial Spearman's validation (Supplementary Figure 2c,d), the *SREBF1* expression was

322   positively associated with *NOTCH1* and *ABCA1*, which we had found to be negatively

323   associated with *APOAB1P*. Contrary to *SREBF1* results, we did not obtain a significant

324   O-PLS between the expression of *SREBF2* and Notch pathway genes (Q^2Y=-0.21).

325   Finally, Aan O-PLS regression model between cholesterol synthesis pathway genes and

326   *NOTCH2NL* identified a negative association with *APOA1BP*, whereas *ABCA1* had the

327   strongest positive association (Supplementary Figure 2e,f).

ASSOCIATIONS OF THE *APOA1BP-SREBF-NOTCH* AXIS WITH THE SERUM METABOLIC PROFILES

330   O-PLS regression models were built to identify serum metabolites associated with the

331   expression of *APOA1BP, SREBF1,* and *SREBF2*. A significant O-PLS model was

332   obtained for the prediction of *APOA1BP* expression levels from the serum metabolic

333   profile (Figure 2a). Significant identified metabolites (Figure 2b) were further validated

334   by partial Spearman's correlation (Figure 2c). We identified asparagine, glycerol,

335   histidine, glycine, choline and citrate as positively associated with the expression of

336   *APOA1BP*, whereas glyceryl of lipids and very low level cholesterol in VLDL were

337   associated negatively. Metabolites associated positively with *APOA1BP* had negative

338   associations with atherosclerosis measures, particularly LmCCA (Figure 2g-i). We also

339   found a borderline significant negative ($r = -0.22$, $P = 0.051$) association between the

340   expression of *APOA1BP* and the inflammatory marker NAG. Metabolome-wide

341   association studies (MWAS) were also performed for the liver expression of *SREBF1* and

15

342 *SREBF2* using O-PLS multivariate regressions (Figure 2d,e and Supplementary Figure

343 3a) confirmed by partial Spearman's correlation (Figure 2 f and Supplementary Figure

344 3b). As expected, the expression of both genes was positively associated with several

345 lipids. They also had positive associations with inflammatory markers [N-

346 acetylglycoproteins (NAG)], branched-chain amino acids (BCAA) (valine, isoleucine)

347 and related catabolites (α-ketoisovalerate), lactate and proline. Most of these metabolites

348 had positive correlations with cIMT measures. In particular, BCAA had positive

349 associations with RICAe (Figure 2j-l), whereas NAG and α-ketoisovalerate had positive

350 associations with mCA (R=0.27, P=0.028; and R=0.33, P=0.006, respectively).

351

352 **ASSOCIATIONS OF HAEMATOPOIESIS AND LIPIDS WITH THE SERUM**

353 **METABOLIC PROFILES**

354 O-PLS regression models were also constructed to reveal associations between those

355 haematological and lipid parameters associated with the *APOA1BP-SREBF-NOTCH* axis

356 and the serum metabolome. A significant model with a strong predictive ability (Figure

357 3a) was obtained between the serum metabolic profile and the HDL-C levels. After

358 validation of the multivariate-identified metabolites by partial Spearman correlation,

359 metabolites associated with HDL-C were similar to those linked to *APOA1BP* and

360 *SREBF1* (Figure 3b). Hence, phosphocholine, choline, glycerol, and glycine, which

361 correlated positively with the expression of *APOA1BP*, also had a positive correlation

362 with HDL-C. Conversely, several lipids, inflammation-related metabolites (NAG),

363 BCAA (valine, isoleucine) and lactate, which were positively associated with *SREBF1*,

364 had a negative association with the circulating levels of HDL-C. Notably, the expression

365 of *NOTCH2NL* correlated negatively with the serum choline and phosphocholine levels

(Figure 3c,d) but positively with the BCAAs leucine and isoleucine (Figure 3e,f). It also had a trend towards a positive association with NAG ($r = 0.20$, $P = 0.07$). A significant O-PLS regression model was also obtained between the fasting serum TG levels and the serum metabolome (Figure 3g). Patients with higher TG concentrations had higher levels of several lipids, BCAAs and catabolites (isoleucine, α-ketoisovalerate), inflammation-related metabolites (NAG), aspartate, proline, and acetone (Figure 3h). As expected, these results agree with those metabolites positively associated with *SREBF1*. Interestingly, several metabolites negatively associated with TG, including glycine, asparagine, phosphocholine, glycerol, and histidine, were found to be positively associated with the expression of *APOA1BP*. Finally, O-PLS regression models (Figure 3i) confirmed by partial Spearman's correlation (Figure 3j) revealed that serum metabolites positively associated with the leukocyte counts were similar to those associated with the expression of *SREBF1*.

Discussion

Atherosclerosis and its progression is caused by lipid accumulation and local inflammation of blood vessels, and is the major underlying cause of CVD. High plasma concentrations of HDL have shown anti-atherogenic potential because HDL carries excess cholesterol away from cells. Importantly, human studies have shown that HDL cholesterol efflux capacity, a metric of HDL function that characterizes a key step in RCT, may be athero-protective [1,3]. Recent *in vitro* and animal studies have shown that AIBP may protect against atherosclerosis [5–8]. Here, we provided evidence for the first time, to our knowledge, in humans of an inverse association between the expression of *APOA1BP* and atherosclerosis measures.

390 *In vitro* studies have demonstrated a role of AIBP in promoting cholesterol efflux from

391 human umbilical vein endothelial cells to HDL and THP-1-derived macrophages, thereby

392 reducing lipid accumulation [5,6]. Mechanistically, AIBP enhances cholesterol efflux and

393 RCT by preventing ABCA1 protein degradation through facilitating its binding to

394 APOA1 on the cell membrane, thereby increasing ABCA1 levels [5]. Growing evidence

395 suggests that ABCA1 protects from atherosclerosis by exporting excess cholesterol from

396 cells to poorly lipidated APOA1, which is essential for the biogenesis of nascent HDL in

397 hepatocytes [23]. Recent animals studies have also highlighted the protection of AIBP

398 against atherosclerosis [7,8]. Hence, *Apoe*^{-/-} mice with established atherosclerosis and

399 treated with recombinant adeno-associated virus (rAAV) to overexpress AIBP showed

400 reduction of atherosclerotic plaque size and inflammation but increased circulating HDL

401 levels and RCT to the liver [8]. In the latter model, AIBP was overexpressed in the aorta

402 and peritoneal macrophages, but mainly in the liver. The striking increase in ABCA1

403 protein levels in the aortas and peritoneal macrophages of AIBP-treated mice suggests

404 again that the effects of AIBP are mediated through ABCA1. In another animal study,

405 *Apoa1bp*^{-/-}*Ldlr*^{-/-} mice fed a high-fat, high-cholesterol diet had exacerbated weight gain,

406 liver steatosis, hyperlipidaemia and atherosclerosis compared to *Ldlr*^{-/-} mice [7]. In

407 addition, AAV-mediated overexpression of AIBP in *Ldlr*^{-/-} mice protected against weight

408 gain, plasma lipid increase and atherosclerosis compared to controls.

409 In agreement with these results, we found negative associations between *APOA1BP*

410 expression levels and measures of cIMT. Conversely, we found a negative association

411 between the expression levels of *APOA1BP* and *ABCA1*. It is possible that in patients

412 with reduced hepatic *APOA1BP* expression, there is a compensatory response in the

413 expression of *ABCA1* to increase cholesterol efflux in the liver. In addition, although

414 treatment with AIBP increased ABCA1 protein levels in macrophages and *Apoe*^{-/-} mice,

415   it did not alter the ABCA1 mRNA expression [5,8]. In fact, ABCA1 protein levels and

416   mRNA expression are usually discordant as ABCA1 protein levels are regulated by a

417   diverse posttranscriptional mechanism [24]. In contrast to ABCA1, which is important

418   for the generation of nascent HDL, scavenger receptor class B member 1 (SR-BI) is a

419   receptor for mature HDL and mediates selective uptake of HDL cholesteryl esters into

420   the liver as the final step in RCT [25]. Consistently, we found a significant association

421   between *APOA1BP* and *SCARB1,* which encodes the SR-BI protein, which was opposite

422   to the *APOA1BP-ABCA1* correlation.

423   In addition to the ABCA1 transporter pathway, the VLDL-APOB secretion pathway has

424   been proposed as the major pathway for the secretion of cholesterol from hepatocytes

425   together with TG into plasma [26]. Interestingly, the VLDL secretion pathway is

426   modulated by SREBP1c [27], encoded by *SREBF1*, which we found strongly positively

427   associated with atherosclerosis. Specifically, *SREBF1* correlated with cIMT measures in

428   all segments of the left carotid artery, which is in agreement the higher vulnerability of

429   the left carotid artery to atherosclerosis [28,29]. Hepatic expression of SREBP1c is also

430   increased in hepatic steatosis [30], which is consistent with our results. A recent study in

431   zebrafish has shown that Aibp2-mediated HPSC expansion through the up-regulation of

432   *Srebf2* (but not *Srebf1*), which predominantly regulates cholesterol synthesis [14].

433   Although we mainly found *SREBF1* associated with cIMT rather than *SREBF2*, it is worth

434   noting that *SREBF1* is transcribed into two variants: SREBP-1c, which solely regulates

435   lipid synthesis, and SREBP-1a, which controls both cholesterol and lipid synthesis. AIBP

436   also seem to modulate Notch signalling in mice and zebrafish [13,14]. In agreement with

437   these results, we found strong associations between the expression of *APOA1BP* and the

438   expression of all Notch signalling receptors and jagged ligands, but not with delta-like

439   ligands. Interestingly, jagged and delta-like ligands have shown opposite effects on

440 angiogenesis and regulation of T cells [31,32]. Consistent with a higher susceptibility of

441 the left carotid artery, w~~Although wc~~e also found that *NOTCH1* and *NOTCH4* were

442 positively associated with left cIMT measures~~atherosclerosis~~. Despite these associations,

443 ~~,~~ it is particularly noticeable that the expression of human-specific gene *NOTCH2NL* had

444 the strongest associations with atherosclerotic measures. Interestingly, among all genes

445 involved in cholesterol metabolism, *NOTCH2NL* had the strongest positive association

446 with *ABCA1*, suggesting again a potential upregulation of *ABCA1* expression to increase

447 cholesterol efflux in patients with cIMT. Little is known about *NOTCH2NL*, but it has

448 recently stood out for its ability to enhance Notch signalling and expand human cortical

449 progenitor cells [33,34]. Consistent with this link between cholesterol metabolism and

450 haematopoiesis, we found significant associations among the expression of *APOA1BP,*

451 *SREBF1* and *NOTCH2NL,* and haematology and lipid parameters.

452 We found that the expression of transcripts *APOA1BP, SREBF1, SREBF2* and

453 *NOTCH2NL* was associated with several plasma metabolites. *APOA1BP* had positive

454 correlations with three amino acids (asparagine, histidine, glycine), glycerol, choline and

455 citrate. These amino acids were also associated positively with circulating HDL-C levels,

456 but negatively with TG and WBC counts. Glycine, histidine and asparagine were also

457 negatively associated with cIMT measures. Of note, among 35 plasma metabolites

458 quantified in 1049 individuals without coronary artery disease (CAD) and diabetes,

459 histidine was most strongly associated with lower risk for incident CAD, followed by

460 asparagine [35]. Low circulating levels of glycine have also been causally associated with

461 higher incidence of coronary heart disease [36].

462 *APOA1BP* expression also had a positive association with choline. Remarkably, both

463 choline and phosphocholine levels had very strong positive associations with HDL-C but

464 correlated negatively with the expression of *NOTCH2NL*. There is evidence suggesting a

20

connection between choline and HDL metabolism. Hence, phosphatidylcholine is the major phospholipid component of all plasma lipoproteins and the biosynthesis of phosphatidylcholine in the liver is critical for the synthesis and secretion of HDL and VLDL [37]. Interestingly, *APOA1BP* correlated negatively with the expression of liver enzymes involved in the synthesis of phosphatidylcholine from choline and phosphocholine, suggesting an up-regulation of the synthesis of phosphatidylcholine in patients with lower *APOA1BP* to increase assembly and clearance of lipoproteins. Due to the role of lecithin–cholesterol acyltransferase (LCAT) in the formation of HDL and RCT, we also assessed the relation between the expression levels of *APOA1BP* and *LCAT* and found a positive association (R=0.27, P=0.01). Interestingly, although studies in the general population gave inconsistent results, patients with LCAT deficiency have shown a significant increase in the incidence of CVD [38]. Despite the associations with *APOA1BP* and HDL, neither choline nor phosphocholine had associations with measures of atherosclerosis. Previous studies have shown inconsistent associations between circulating choline and phosphocholine levels and cardiovascular events. Both metabolites were positively associated with the risk of CVD and stroke after 4.8 years of follow-up in participants at high cardiovascular risk within the PREDIMED study, but no associations were found between 1-year changes in these metabolites and the incidence of CVD [39]. Similarly, higher plasma choline levels were associated with increased risk of major cardiac events after 3 years of follow-up in patients undergoing elective diagnostic coronary angiography, but they did not predict risk when trimethylamine *N*-oxide, a choline metabolite, was added to the model [40]. In cross-section studies higher plasma choline concentrations have been associated with an unfavourable cardiometabolic risk factor profile, including lower HDL levels [41,42]. However, it is worth noting that plasma choline levels do not reflect choline status because disturbances

21

490　in circulating choline arise early in disease development [43,44] and circulating levels

491　may depend on lipid overload. In this sense, plasma choline metabolites differed between

492　normal and overweight men [45]. In addition, both low and high plasma concentrations

493　may be associated with adverse effects [46].

494　As expected, the expression levels of *SREBF1* and *SREBF2* had positive associations with

495　several lipids. These genes also correlated positively with circulating levels of BCAA and

496　catabolites, NAG and lactate. Except for lactate, we found that these metabolites were all

497　positively associated with measure of cIMT. Several cross-section and prospective cohort

498　studies revealed positive associations of BCAA with major metabolic disorders [47] and

499　recently they have also been positively associated with risk factors of CAD, in particular

500　cIMT [48]. Interestingly, we also found a positive correlation between the expression

501　levels of *NOTCH2NL* and BCAA. In line with atherosclerosis being a chronic

502　inflammatory disorder, we found a consistent association between NAG, a novel marker

503　of chronic inflammation [22], and cIMT measures. Consistently, we found that NAG

504　levels were the strongest predictors of WBC counts, which also had positive associations

505　with BCAA. Supporting the role of the *APOA1BP-SREBF-NOTCH* axis and

506　inflammation in atherosclerosis, we also found that NAG levels were negatively

507　associated with the expression of *APOA1BP*, but positively with that of *SREBF1*,

508　*SREBF2, and NOTCH2NL.* Finally, most of the metabolites associated with *SREBF1* and

509　*SREBF2* had similar associations with TG, but opposite to those of HDL.

510　The main limitation of our study is its cross-sectional nature. Therefore, we cannot infer

511　cause-effect relationships. Second, the results cannot be extrapolated to the general

512　population, as subjects in the current study were morbidly obese. In addition, we did not

513　measure AIBP protein levels. The strengths of the present work include the use of two

514　independent well-characterized cohorts, which allowed the control for potential

515 confounders, with comprehensive metabolomic, transcriptomic, liver biopsy and cIMT

516 data.

517 In conclusion, our findings demonstrate, for the first time, an atheroprotective association

518 between the expression levels of *APOA1BP* and atherosclerosis in humans. We have also

519 shown a connection between *APO1ABP,* Notch signalling and inflammation with

520 atherosclerosis.

521

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690

691 *Figure Legends*

692 **Figure 1. Associations of expression levels of *APOA1BP, SREBFs,* and *NOTCHs* gens**

693 **with cIMT, steatosis, haematology and lipid measures and cholesterol and notch**

694 **pathway genes. a)** Heatmap displaying the partial Spearman's correlation coefficients

695 (adjusted for age, BMI, sex, and cohort) between genes and cIMT measures. *$p<0.05$,

696 **$p<0.01$, ***$p<0.001$. **b)** Association of expression levels of *SREBF1* with the steatosis

697 degree (ANCOVA and Tukey-kramer tests). **c)** Cholesterol metabolism pathway genes

698 significantly associated with mean CCA based on partial Spearman's correlation adjusted

699 for age, BMI, sex, and cohort. **d-i)** Scatter plots between expression levels of selected

700 genes and haematological and lipid parameters. Partial Spearman's correlation and

701 significance values adjusted for age, BMI, sex, and cohort are shown. **j-k)** Permutation

702 test for the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-

703 PLS models between the expression levels of *APOA1BP* and the expression levels of

704 genes involved in the Notch signalling and cholesterol pathways, respectively. **l-m)**

705 Significant Notch and cholesterol pathway genes associated with *APOA1BP* after further

706 validation of the O-PLS identified genes by partial Spearman's correlation adjusting for

707 age, sex, BMI, and country.

708 **Figure 2. Associations of metabolomics data with the expression levels of *APOA1BP,***

709 ***SREBF1,* and cIMT measures. a)** Permutation test for the goodness-of-fit (R^2Y) and

710 goodness of prediction (Q^2Y) obtained from the O-PLS models between the expression

711 levels of *APOA1BP* and the serum metabolome. **b)** Significant serum metabolites

712 obtained from the O-PLS model. Statistically significant metabolites are coloured in red

713 if positively associated with *APOA1BP* and blue if negatively associated. **c)** Metabolites

714 associated with *APOA1BP* after further validation of the O-PLS identified metabolites by

715 partial Spearman's correlation adjusting for age, sex, BMI, and country. **d)** Permutation

32

716 test for the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-

717 PLS models between the expression levels of *SREBF1* and the serum metabolome. **e)**

718 Significant serum metabolites obtained from the O-PLS model. Statistically significant

719 metabolites are coloured in red if positively associated with *SREBF1* and blue if

720 negatively associated. **f)** Metabolites associated with *SREBF1* after further validation of

721 the O-PLS identified metabolites by partial Spearman's correlation adjusting for age, sex,

722 BMI, and country. **g-l)** Scatter plots between selected metabolites and cIMT measures.

723 Partial Spearman's correlation and significance values adjusted for age, BMI, sex, and

724 cohort are shown.

725 **Figure 3. Associations of metabolomics data with the lipid parameters, WBC, and**

726 ***NOTCH2NL* expression levels. a)** Permutation test for the goodness-of-fit (R^2Y) and

727 goodness of prediction (Q^2Y) obtained from the O-PLS models between the expression

728 levels of HDL-C and the serum metabolome. **b)** Metabolites associated with HDL-C after

729 further validation of the O-PLS identified metabolites by partial Spearman's correlation

730 adjusting for age, sex, BMI, and country. **c-f)** Scatter plots between selected metabolites

731 and *NOTCH2NL* expression levels. Partial Spearman's correlation and significance

732 values adjusted for age, BMI, sex, and cohort are shown. **g)** Permutation test for the

733 goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-PLS models

734 between the expression levels of TG and the serum metabolome. **h)** Metabolites

735 associated with TG after further validation of the O-PLS identified metabolites by partial

736 Spearman's correlation adjusting for age, sex, BMI, and country. **i)** Permutation test for

737 the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-PLS

738 models between the expression levels of WBC and the serum metabolome. **j)** Metabolites

739 associated with WBC after further validation of the O-PLS identified metabolites by

740 partial Spearman's correlation adjusting for age, sex, BMI, and country.

Supplementary Figure 1. **a)** Cholesterol metabolism pathway genes significantly associated with mean CA based on partial Spearman's correlation adjusted for age, BMI, sex, and cohort. **b)** Heatmap displaying the partial Spearman's correlations (adjusted for age, BMI, sex, and cohort) between cIMT measures and inflammation measured by NMR plasma levels of N-acetylglycoproteins (NAG). **c)** Heatmap displaying the partial Spearman's correlation coefficients (adjusted for age, BMI, sex, and cohort) between genes and haematological parameters. **d)** Heatmap displaying the partial Spearman's correlation coefficients (adjusted for age, BMI, sex, and cohort) between haematological parameters and cIMT measures.

Supplementary Figure 2. **a,b)** Permutation test for the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-PLS models between the expression levels of *SREBF1* and the expression levels of genes involved in Notch signalling and cholesterol synthesis pathways, respectively. **c,d)** Significant Notch signalling and cholesterol pathway genes associated with *SREBF1* after further validation of the O-PLS identified genes by partial Spearman's correlation adjusting for age, sex, BMI, and country, respectively. **ea)** Permutation test for the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-PLS models between the expression levels of *NOTCH2NL* and the expression levels of genes involved in cholesterol synthesis pathway. **fb)** Significant cholesterol pathway genes associated with *NOTCH2NL* after further validation of the O-PLS identified genes by partial Spearman's correlation adjusting for age, sex, BMI, and country.

Supplementary Figure 3. **a)** Permutation test for the goodness-of-fit (R^2Y) and goodness of prediction (Q^2Y) obtained from the O-PLS models between the expression levels of *SREBF2* and the serum metabolome. **b)** Significant serum metabolites obtained from the O-PLS model. Statistically significant metabolites are coloured in red if positively

766 associated with *SREBF2* and blue if negatively associated. **c)** Metabolites associated with

767 *SREBF2* after further validation of the O-PLS identified metabolites by partial

768 Spearman's correlation adjusting for age, sex, BMI, and country

769

770 **Table 1.** Baseline characteristics of participants.

Variables	All (*n*=78)
Age (years)	42.4 ± 1.16
BMI (kg/m^2)	46.2 (42.4-51.1)
Waist circumference (cm)	127.0 (119.5-138.0)
Sex (women, %)	79.2
SBP (mmHg)	134.0 (122.5-144.0)
DBP (mmHg)	81.0 (75.0-90.0)
Biochemistry	
Glucose (mg/dL)	95.0 (88.5-101.5)
HOMA-IR	4.50 (2.95-6.90)
Triglycerides (mg/dL)	112.0 (82.0-143.5)
Total cholesterol (mg/dL)	197.5 ± 4.6
LDL cholesterol (mg/dL)	131.0 ± 4.0
HDL cholesterol (mg/dL)	45.0 (40.0-51.2)
Haematology	
Eosinophils	150.0 (100.0-200.0)
Neutrophils	4810 (3665-5795)
Lymphocytes	2360 ± 86.9
Monocytes	500 (420-600)
Total WBC	7350 (6385-9030)
RBC	4.66 (4.46-4.91)
Liver	
HsCRP	0.88 (0.47-1.39)
AST	19.0 (15.0-28.0)
ALT	30.0 (22.0-43.5)
Steatosis grade (%):	
Grade 0	15.6
Grade 1	33.8
Grade 2	24.7
Grade 3	26.0
Atherosclerosis	
RproCCAi	0.70 (0.60-0.80)
RproCCAe	0.70 (0.60-0.80)
RpreCCAi	0.79 ± 0.03
RpreCCAe	0.72 (0.60-0.80)
RICAi	0.70 (0.60-0.90)
RICAe	0.70 (0.54-0.85)
mRCCA	0.73 ± 0.02
mRICA	0.71 ± 0.02
LproCCAi	0.70 (0.60-0.85)
LproCCAe	0.69 (0.60-0.80)
LpreCCAi	0.80 (0.70-1.0)
LpreCCAe	0.84 ± 0.03
LICAi	0.77 (0.57-0.90)
LICAe	0.70 (0.60-0.87)
mLCCA	0.76 ± 0.02
mLICA	0.73 ± 0.03
mCCA	0.74 ± 0.02
mCA	0.73 ± 0.02

771 Values are expressed as means ± SEM for normally distributed variables and median [IQR] for non-normally
772 distributed variables. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HsCRP, high sensitivity

773   C-reactive protein; e, external; i, internal; ICA, internal carotid artery; L, left; m, mean; preCCA, pre bifurcation
774   common carotid artery; proCCA, proximal segment common carotid artery; R, right; RBC, red blood cells; WBC,
775   white blood cells.

Hospital Universitari de Girona
Doctor Josep Trueta

IdIBGi Institut
d'Investigació
Biomèdica
de Girona
Dr. Josep Trueta

1   Reference: YCLNU-D-19-01600
2
3   Title: **The APOA1BP-SREBF-NOTCH axis is associated with reduced atherosclerosis**
4   **risk**

5   **General comments to the reviewers**

6   We greatly appreciate all the comments and suggestions made by the reviewers, the
7   responses to which strengthen our manuscript. We have revised and modified the
8   manuscript according to these suggestions, as detailed below.

9
10   **Responses to Reviewer 1**
11
12   **Reviewer #1:** *The manuscript titled "The APOA1B-SREBF-NOTCH axis is associated with*
13   *reduced atherosclerosis risk," is interesting and clearly written. It has a novelty because*
14   *the authors tried to elucidate the association between APOA1B-SREBF-NOTCH axis and*
15   *atherosclerosis in humans. However, the reviewer has several concerns regarding this*
16   *article. Specific comments are as follows.*
17
18   *Major points:*
19
20   *1.   The authors demonstrated the possible relations between APOA1B and SREBF, and*
21   *between APOA1B and NOTCH. However, the reviewer cannot find the data showing the*
22   *relation between SREBF-NOTCH. Since the authors claim "the APOA1B-SREBF-NOTCH*
23   *axis", they should show clearly the relation between SREBF-NOTCH.* **We thank the**
24   **reviewer for this comment and we agree with him that we had not shown a clear**
25   **evidence for the relation between *SREBF* and *NOTCH* signalling. Therefore, similar**
26   **to what we did for the associations between *APOA1BP* expression and Notch and**
27   **cholesterol pathway associated genes, we built O-PLS models between the expression**
28   **of *SREBF1* and Notch and cholesterol genes, respectively. In both cases, we obtained**
29   **significant models ($P<0.001$) associating *SREBF1* with Notch and cholesterol**

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pathways. Both models were further validated by partial Spearman's correlation
adjusting for age, sex, BMI, and country. Interestingly, the expression of *SREBF1*
was positively associated with both *NOTCH1* and *ABCA1* expression, which we had
found negatively associated with *APOA1BP*. In the case of *SREBF2*, however, we did
not find significant associations with the Notch pathway genes. We have added all
these results in the results section and we have created a new Supplementary Figure
2.

2.   In this study, the authors investigate morbidly obese population only, however, the
title does not imply this specific population. The reviewer feels this could be misleading for
the readers. **We agree with the reviewer that the title lacks information about the**
study population and it can be misleading. We have added this information in the
title and now it reads as: "The APOA1BP-SREBF-NOTCH axis is associated with
reduced atherosclerosis risk in morbidly obese patients".

Minor points:

1.   The O-PLS regression model figures are difficult to understand because those figures
do not have adequate variables information. Please refer to figure 1j-k, 2a-b, 2d-e, 3a, 3g,
3i, Supplemental figure 2a and Supplemental figures 3a-b. It would be easier to
understand if these figures contain adequate information regarding the variables. **We**
agree with the reviewer that the figures are no straightforwardly understandable
without reading the figure legends. Following reviewer's suggestion, we have added
in each figure the variables X and Y involved in the corresponding model.

2.   The reviewer found mistyping which is mentioned below:
Page 13, line 280, "RproCAAi" should be "RproCCAi". **Corrected**
Page 22, line 507, "APO1BP" should be "APOA1BP". **Corrected**

60   **Responses to Reviewer 2**

61

62   *Reviewer #2:* *Mayneris-Perxachs, et al. investigated correlation between gene expression*

63   *in the liver (e.g., APOA1BP, SREBF, and Notch) and carotid atherosclerosis in morbidly*

64   *obese patients. The authors employed sophisticated methods such as metabolomics*

65   *analyses and transcriptomic analyses, and suggested "APOA1BP-SREBF-NOTCH axis*

66   *signaling pathway" and its association with atherosclerosis.*

67   *This reviewer has following comments to this study.*

68   *1.   The mechanisms by which APOA1BP-SREBF-NOTCH axis signaling pathway affect*

69   *atherogenesis is not clear. How the difference in gene expression in the liver links with the*

70   *carotid artery atherosclerosis?*

71   **Recent evidence suggests that cholesterol pathways link hematopoiesis with**

72   **atherosclerosis, with and interesting role for the AIBP-SREBP-NOTCH axis. The**

73   **bone marrow is the main site of haematopoiesis in adults. However, the liver is the**

74   **main site in prenatal development and some studies have provided evidence that it is**

75   **still active in adults. Therefore, we hypothesized that the gene expression in the liver**

76   **could be related to cholesterol metabolism and atherosclerosis. Thus, our aim was to**

77   **show for the first time in humans an association of the *APOA1BP-SREBF-NOTCH***

78   **axis with atherosclerosis. We have to take into account that we have analysed cross-**

79   **sectional data. Therefore, we cannot infer causality or describe mechanisms, but just**

80   **associations. In zebrafish, increased cholesterol efflux mediated by Aibp2 has shown**

81   **to activate Srebp2, which in turn upregulated Notch resulting in an expansion of**

82   **hematopoietic stem and progenitor cells (HPSC) in the bone marrow. In human**

83   **subjects with hypercholesterolemia (the driving force for atheroscleroris) they found**

84   **that the number of HPSC are elevated and SREBP and NOTCH are upregulated in**

85   **HPSC isolated from these subjects. Here, we also found positive associations between**

86   ***SREBF1, SREBF2* and *NOTCH2NL* expression and atherosclerosis parameters and**

87   **blood cells, whereas *APOA1BP* had opposite associations. We also found associations**

88   **among *APOA1BP,* Notch pathway genes, and cholesterol pathway genes. However,**

89   **we had not reported associations between *SREBF* and Notch pathway and cholesterol**

90   **pathway genes. We have thus analysed this relationship by O-PLS modelling and**
91   **similar to *APOA1BP* we have found significant models between *SREBF1* and Notch**
92   **and cholesterol genes, respectively. We have added these results in the revised version**
93   **of the manuscript. Therefore, lower *APOAB1P* expression is associated with higher**
94   **expression of *SREBF1* and *NOTCH* receptors, which in turn are associated with**
95   **higher levels of blood cells and atherosclerosis measures. We have also found**
96   **significant associations between several white blood cells and atherosclerosis**
97   **measures. Although we had not reported these associations in the original**
98   **manuscript, we have added them in supplementary Figure 1d. Thus, we found a clear**
99   **association among the *APOA1BP-SREBF-NOTCH* axis, haematopoiesis and**
100   **atherosclerosis.**

101

102   *2.   Please explain the difference in the correlation between gene expression (e.g.,*
103   *APOA1BP, SREBF, and NOTCH) and parameters for atherosclerosis in left/right carotid*
104   *arteries (Page13).*

105   *APOA1BP* **was negatively associated with measurements in both left and right**
106   **carotid arteries. Interestingly, *SREBF1* was mainly positively associated with all the**
107   **measurements in the left carotid artery, which would be in agreement with recent**
108   **evidence that the left carotid artery is more vulnerable to atherosclerosis than the**
109   **right carotid artery [1,2]. This is also consistent with NOTCH1 and NOTCH4**
110   **positively associated with atherosclerosis measures in the left carotid artery. We have**
111   **added this information in the discussion. In the case of *NOTCH2NL* though, it**
112   **correlated with measures of the right internal carotid artery, but the reason for this**
113   **association would need further investigations.**

114   [1]   Selwaness M, Van Den Bouwhuijsen Q, Van Onkelen RS, Hofman A, Franco OH,
115   Van Der Lugt A, et al. Atherosclerotic plaque in the left carotid artery is more
116   vulnerable than in the right. Stroke 2014;45:3226–30.
117   doi:10.1161/STROKEAHA.114.005202.
118   [2]   Chou CL, Wu YJ, Hung CL, Liu CC, Wang S De, Wu TW, et al. Segment-specific
119   prevalence of carotid artery plaque and stenosis in middle-aged adults and elders in

120   Taiwan: A community-based study. J Formos Med Assoc 2019;118:64–71.

121   doi:10.1016/j.jfma.2018.01.009.

122

123   *3.   How do "APOA1BP", "SREBF", and "NOTCH" interact each other?*

124   **From O-PLS modelling and further validation by partial Spearman correlation**

125   **(adjusting for age, BMI, sex, and country) we had found significant associations**

126   **between the expression of *APOA1BP* and NOTCH pathway genes. We also showed**

127   **that *APOA1BP* and *SREBF1* had opposite associations with the almost the same**

128   **atherosclerosis measures, particularly mLCCA, mCCA, and mCA. However, we had**

129   **not shown a direct relationship between *SREBF* and *NOTCH*. Thus, similar to what**

130   **we did for the associations between *APOA1BP* expression and Notch and cholesterol**

131   **pathway associated genes, we built O-PLS models between the expression of *SREBF1***

132   **and Notch and cholesterol genes, respectively. In both cases, we obtained significant**

133   **models ($P<0.001$) associating *SREBF1* with Notch and cholesterol pathways. Both**

134   **models were further validated by partial Spearman's correlation. Interestingly, the**

135   **expression of *SREBF1* was positively associated with both *NOTCH1* and *ABCA1***

136   **expression, which we had found negatively associated with *APOA1BP*. In the case of**

137   ***SREBF2*, however, we did not find significant associations with the Notch pathway**

138   **genes. We have added all these results in the results section and we have created a**

139   **new Supplementary Figure 2. Therefore, we have found a clear relationship between**

140   ***APOAB1P, SREBF,* and *NOTCH,* but due to the cross-sectional nature of our study**

141   **we cannot infer causal mechanisms or the direction of the associations.**

142

143   *4.   The authors only described about Jagged ligand related with Notch. A previous study*

144   *demonstrated the inhibition of Dll4, a Notch ligand, reduced lipid accumulation in the*

145   *liver (PNAS 2012;109:E1868-E1877.).*

146   **Thanks for pointing this. In Figure 1l we only showed the significant associations that**

147   **we obtained between *APOA1BP* and genes from the Notch pathway. However, the O-**

148   **PLS models were performed considering 79 genes involved in the Notch signalling**

149   **pathway. Then, significant genes identified through O-PLS modeling were further**

150   validated by partial Spearman's correlation. These 79 genes included both jagged

151   and delta-like ligands, but we did not find significant associations between delta-like

152   ligand and *APOA1BP* expression. We only found significant associations with jagged

153   ligands. Because of this, we have not discussed delta-like ligands in the manuscript.

154   To avoid confusions, we have added the number of genes that we considered to build

155   the O-PLS models based on Notch signalling pathway associated genes and

156   cholesterol pathway associated genes in the revised version of the manuscript. We

157   have also added the names of these genes in the Supplementary Tables 1 and 2,

158   respectively.

159

160   On the other hand, in our liver samples, only *DLL1, DLK1 (*delta like non-canonical

161   Notch ligand1)*, and DLK2* were expressed, but not *DLL4*. Considering Fukuda *et al.*

162   results suggested by the reviewer, we have analysed the possible association between

163   delta-like ligands and steatosis. Thus, we have performed partial Spearman's

164   correlation analysis between these delta ligands and the steatosis degree in our

165   population. However, we have not found any significant association, although the

166   expression of *DLK1* had a trend towards a positive association with the degree of

167   steatosis (r=0.21, p=0.076). We must also take into account that Fukuda *et al.* treated

168   mice with anti-mouse *Dll4* antibody, which inhibits *Dll4* in all *Dll4*-expressing cells,

169   not just the liver. Therefore, decreases in the accumulation of lipids in the liver after

170   *Dll4* Ab treatment could originate from blockage of *Dll4* in other tissues. In fact, *Dll4*

171   Ab treatment also reduced fat in epididymal and sub-cutaneous adipose tissues.

172

173   *5. Do the authors have data associated with inflammation directly?*

174   From multivariate analyses, we have found direct associations of *SREBF1* and

175   *SREBF2* expression with *N*-acetylglycoproteins (NAG) measured by NMR. It is a

176   composite biomarker of systemic inflammation that integrates the protein levels and

177   glycosylation states of several of the most abundant acute phase proteins in serum. As

178   a composite biomarker, NAG is a novel marker of systemic inflammation that may be

179   a better reflection of systemic acute phase response than any other single

180   glycoprotein component. For example, it has low intra-individual variability
181   compared to hsCRP, which often exhibits high intra-individual variability, allowing a
182   more stable measure of inflammation.

183

184   We have now specifically analysed the correlation between NAG and *APOA1BP* and
185   *NOTCH* receptors. We have found an almost significant association between NAG
186   and *APOA1BP* (r=-0.22, p=0.051). We have also found a trend towards a positive
187   association between *NOTCH2NL* and NAG (r=0.20, p=0.07), but no significant
188   associations with the expression of the other Notch receptors. We have added these
189   results in the results section.

190

191   We have also realized that although in lines 491-493 we had commented that NAG
192   was associated with cIMT measures, we had not reported the associations in the
193   results section. Therefore, we have added a heatmap in Supplementary Figure 1b
194   with the correlations between NAG and measures of atherosclerosis. As expected,
195   several of these measures correlated positively with NAG.

196

197   *6. Can the authors show the correlation between gene expression (e.g., APOA1BP,*
198   *SREBF, and NOTCH) and WBC population such as monocyte or lymphocytes?*
199   We have added a heatmap in Supplementary Figure 1c with the correlations of
200   haematological parameters (total WBC, lymphocytes, monocytes, eosinophils,
201   neutrophils, red blood cells, and platelets) with the expression *APOAB1P*, *SREBF*
202   and *NOTCH* genes.

203

204   *7. Can the author apply the results of present study to general population? If not, the*
205   *authors need to make this clear in the title of this paper.*
206   As our population consisted in morbidly obese patients, the results may not be
207   generalizable to the general population. Following the reviewer's suggestion we have
208   modified the title and now it reads as: "The APOA1BP-SREBF-NOTCH axis is
209   associated with reduced atherosclerosis risk in morbidly obese patients".

Comments to the Editor

We have added Dr. Josep Puig as an author in the paper. He was the person responsible for the measurements of carotid intima-media thickness, but as he has moved to another area we had forgotten to add him as an author. We apologise for this mistake and any inconvenience it may have caused.

Figure1   Click here to access/download;Figure;Figure1_v4.jpg ⬇

Figure2   Click here to access/download;Figure;Figure2_v2.jpg ±

Figure3

Click here to access/download;Figure;Figure3.jpg ±

a Serum metabolome (X) HDL-C (Y); R²Y= 0.65, Q²Y= 0.41, P < 0.001

b HDL-C partial Spearman's correlation — Phosphocholine, Choline, Glycerol, Glycine, Glutamate, Low level cholesterol in LDL, Lipid edge, Valine, Aspartate, Lipids (VLDL,saturated), Lipid unsaturated, NAG, Lactate, Lipid (mostly LDL), VLDL lipid, Glyceryl of lipids, Isoleucine, Acetone, VLDL sat lipid, Lipid

c R=-0.27, P=0.02

d R=-0.30, P=0.008

e R=0.27, P=0.021

f R=0.30, P=0.009

g Serum metabolome (X) TG(Y); R²Y= 0.76, Q²Y= 0.65, P < 0.001

h TG partial Spearman's correlation — VLDL lipid, Lipid (mostly LDL), Glyceryl of lipids, Lipid, VLDL sat lipid, Lipid unsaturated, Lipids (VLDL, saturated), alpha-ketoisovalerate, Acetone, Lactate, Low level chol in LDL, Aspartate, 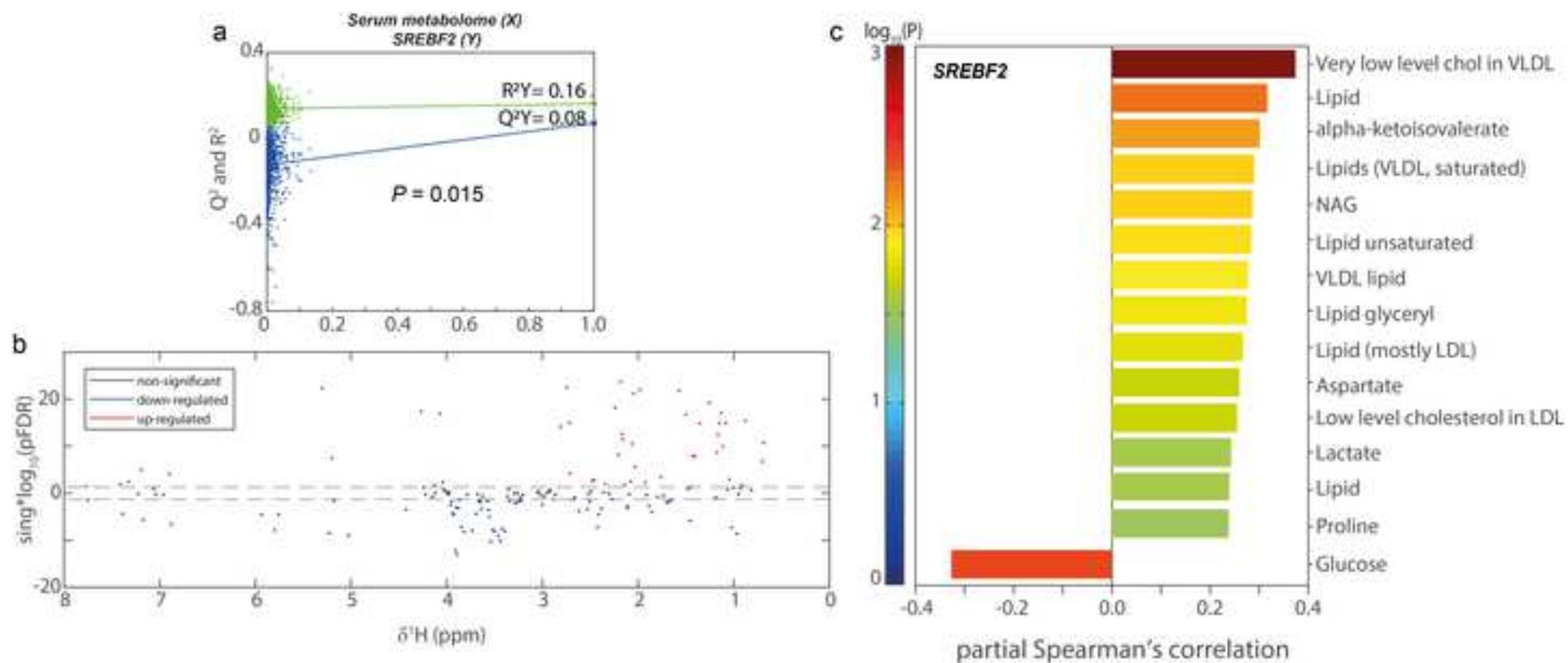NAG, Very low level chol in VLDL, Lipid_edge, Isoleucine, Proline, Creatinine, scyllo-Inositol, Phosphocholine, Acetate, Lysine/Arginine, Tyrosine, Creatine, Asparagine, Succinate, Histidine, Glucose, Glycerol, Glutamine, Glycine, Citrulline

i Serum metabolome (X) WBC(Y); R²Y= 0.15, Q²Y= 0.04, P = 0.025

j WBC partial Spearman's correlation — NAG, Lipid edge, Aspartate, Pyruvate, Proline, Lactate, Glutamate, Isoleucine, Glyceryl of lipids, Lipid, VLDL lipid, Lipid (mostly LDL), Citrulline, Histidine, Glutamine

a

Serum metabolome (X)
SREBF2 (Y)

$R^2Y = 0.16$
$Q^2Y = 0.08$

$P = 0.015$

Q² and R²

b

sing*log₁₀(pFDR)

non-significant
down-regulated
up-regulated

δ¹H (ppm)

c log₁₀(P)

SREBF2

Very low level chol in VLDL
Lipid
alpha-ketoisovalerate
Lipids (VLDL, saturated)
NAG
Lipid unsaturated
VLDL lipid
Lipid glyceryl
Lipid (mostly LDL)
Aspartate
Low level cholesterol in LDL
Lactate
Lipid
Proline
Glucose

partial Spearman's correlation

Supplementary Table 1. Notch signalling pathway associated genes considered in the present study.

Genes
AAK1
ADAM10
AKT1S1
ANXA4
APP
BMP2
CBFA2T2
CDK6
CDKN1B
CIR1
CREB1
CREBBP
CTBP1
CTBP2
DLK1
DLL1
DVL1
DVL3
EP300
EPN1
EPN2
FBXW7
FOXC1
GALNT11
GMDS
GOT1
HDAC1
HDAC2
HES1
HES2
HES4
HES5
HES6
HES7
HESX1
HEY1
HEY2
HHEX
HIF1AN
IFT172
IFT88

JAG1
JAG2
KAT2A
KAT2B
MAML1
MAML2
MIB1
MIB2
MMP14
NCOR2
NCSTN
NEURL
NFKBIA
NLE1
NOTCH1
NOTCH2
NOTCH2NL
NOTCH3
NOTCH4
POFUT1
POGLUT1
PSEN2
PTP4A3
RBM15
RBPJ
RFNG
RPS19
RPS27A
SEL1L
SNAI2
SORBS2
SPEN
TSPAN14
TSPAN15
UBA52
UBB
UBC
WDR12

Supplementary Table 2. Notch signalling pathway associated genes considered in the present study.

Genes
ABCA1
ABCG1
ACADL
ACAT2
ALMS1
APLP2
APOA1
APOA1BP
APOA2
APOA4
APOA5
APOB
APOBEC2
APOBEC3A
APOBEC3B
APOBEC3C
APOBEC3D
APOBEC3F
APOBEC3G
APOBEC3H
APOBR
APOC1
APOC2
APOC3
APOC4
APOE
APOF
APOH
APOL1
APOL2
APOL3
APOL4
APOL5
APOL6
APOLD1
ARHGEF10L
ARV1
CAT
CLN6
CYB5R1
CYB5R3

CYP51A1
DHCR24
DHCR7
EBP
EBPL
EHD1
EIF2A
EIF2AK3
ERLIN1
ERLIN2
FDFT1
FDPS
FDX1
FECH
HDLBP
HMGCR
HSD17B7
INSIG1
INSIG2
IRAK1
LBR
LDLR
LDLRAP1
LIPA
LIPE
LMF1
LMNA
LRP5
LRP6
LSS
MBTPS1
MED13
MVD
MVK
NCOR1
NPC1
NPC1L1
NPC2
NR1H2
NR1H3
NUS1
OSBP
PCTP
PLSCR3
PMVK

POR
PRKAA1
PTCH1
RALY
RORA
SC5DL
SCAP
SCARB1
SCP2
SEC14L2
SEC24A
SIRT1
SOD1
SQLE
SREBF1
SREBF2
STARD4
STARD5
STX12
TM7SF2
TMEM97
VPS4A
XBP1