Differential expression of osteopontin, and osteoprotegerin mRNA in epicardial adipose tissue between patients with severe coronary artery disease and aortic valvular stenosis: association with HDL subclasses

María Luna-Luna¹, David Cruz-Robles¹, Nydia Ávila-Vanzzini², Valentín Herrera-Alarcón³, Jesús Martínez-Reding⁴, Sergio Criales-Vera⁵, Julio Sandoval-Zárate⁶,⁹, Jesús Vargas-Barrón⁵,⁹, Carlos Martínez-Sánchez⁷,⁹, Armando Roberto Tovar-Palacio⁵, José Manuel Fragoso¹,⁹, Elizabeth Carreón-Torres¹,⁹, Gilberto Vargas-Alarcón¹,⁹ and Óscar Pérez-Méndez¹,⁹*  

Abstract  

Background: Previous studies suggest a relationship of the epicardial adipose tissue (EAT) with progression and calcification of the atherosclerotic plaque; however, it is unknown if this tissue expresses genes that may participate on these processes and if the expression of these genes is regulated by high-density lipoprotein (HDL) subclasses.

Methods: To explore this possibility, we determined the mRNA expression by qPCR of a pro-calcifying gene (osteopontin (OPN)), and two anti-calcifying genes (osteoprotegerin (OPG) and osteonectin (ON)), in biopsies of EAT obtained from 15 patients with coronary artery disease (CAD) determined by angiography, and 15 patients with diagnostic of aortic valve stenosis but without CAD as control group. We determined the distribution and composition of HDL subclasses by electrophoresis and their statistical relationship with the gene expression in EAT.

Results: EAT from CAD patients showed a higher expression level of OPN and OPG than control group, whereas ON expression was similar between groups. Large HDL subclasses were cholesterol-poor in CAD patients as estimated by the cholesterol-to-phospholipid ratio. A linear regression model showed an independent association of OPN expression with HDL3a-cholesterol, and OPG expression with the relative proportion of HDL3b protein. Logistic analysis determined that OPN expression was positively associated with the presence of atherosclerotic plaque

Conclusion: OPN, ON, and OPG genes are transcribed in EAT; to the exception of ON, the level of expression was different in CAD patients and control group, and correlated with some HDL subclasses, suggesting a new role of these lipoproteins.

Keywords: Adipose tissue, High-density lipoprotein, Osteopontin, Osteonectin, Osteoprotegerin

*Correspondence: opmendez@yahoo.com  
¹Department of Molecular Biology, Instituto Nacional de Cardiología "Ignacio Chávez", Juan Badiano 1, Sección XVI, 14080 México City, D.F., Mexico  
²Study Group of Atherosclerosis, Instituto Nacional de Cardiología "Ignacio Chávez", México City, Mexico  
Full list of author information is available at the end of the article

© The Author(s). 2017 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background
Epicardial adipose tissue (EAT) is found in the atrio- and inter-ventricular grooves, and closely surrounds the coronary arteries, even invading the adventitia in some sections [1]. Atherosclerotic lesions are present in sections of the artery adjacent to EAT [1–4], whereas these lesions are never found in regions of the artery that sink into the myocardium. These evidences suggest that EAT contributes to the development and calcification of the atherosclerotic plaque, probably interacting with the coronary arteries through a paracrine signaling [2]. Thereby, EAT may be a source of expression of genes that encodes pro- and anti-calcifying proteins such as osteopontin, osteoprotegerin, and osteonectin.

Osteopontin is encoded by OPN gene, and it is a multi-functional protein [5] that promotes the inflammatory micro-environment characteristic of the atherosclerosis [6]. In addition, several studies have demonstrated that osteopontin increases with progression and calcification of the atherosclerotic plaque [6–8], suggesting a pro-calcifying role of this protein.

Osteoprotegerin is encoded by OPG gene and it is member of the family of TNF receptor [9]. Osteoprotegerin has been considered an anti-calcifying protein since it prevents the receptor activator of NF-kB (RANK) binds to its ligand (RANKL) [10]. By this mechanism, osteoprotegerin inhibits the transcription of pro-calcifying genes as bone morphogenetic protein-4 (BMP-4) and alkaline phosphatase (ALPL) [11, 12]. In addition, osteoprotegerin may interact with TNF-related apoptosis inducing ligand (TRAIL) and avoid the formation of anti-apoptotic bodies, which can be mineralized [13–15].

ON gene encodes for osteonectin that has been considered as an anti-calcifying protein since the level of expression of ON decreases with the progression of atherosclerosis and it is lower in calcified atherosclerotic plaques [8].

Several tissues express OPN, OPG, and ON [10, 16–19] but little is known about the expression of these genes in EAT; in this context, the secretion of osteoprotegerin is induced by high-density lipoprotein (HDL) in in vitro cultures [20]. These data suggest that HDLs protect against the progression of atheroma calcification through mechanisms that evoke the regulation of genes related with this process. HDLs constitute a heterogeneous family of lipoproteins that may be classified by size in HDL2b, HDL2a, HDL3a, HDL3b and HDL3c [21, 22]. These subclasses differ in their lipid and protein content, and they may have different anti-atherogenic characteristics [23–25]. In agreement with this idea, a previous report from our group demonstrated that asymptomatic subjects with high coronary calcium score had lower number of HDL particles and a high proportion of small HDL subclasses enriched with triglycerides [26]; HDL subclasses may be indirect players in the calcification process of the atherosclerotic plaque through the regulation of the expression of genes that encode pro- and anti-calcifying proteins.

Therefore, the aim of this study was to determine whether the mRNA expression levels of OPN, OPG and ON in EAT from patients with coronary artery disease (CAD) is different from that of subjects without coronary lesions determined by angiography. We further determined the potential statistical association of mRNA levels in EAT and HDL subclasses.

Methods
Patients
We obtained EAT biopsies from 30 patients, 15 with coronary artery disease (CAD) candidate to revascularization surgery. As comparison (named control) group, we enrolled 15 patients with diagnostic of aortic valve stenosis, without CAD and who were programmed for chirurgical valve replacement; since the etiology of aortic valve stenosis is similar to that of the CAD, this comparison group is of particular relevance to focus on the potential paracrine contribution of EAT to the atherosclerotic lesion.

The presence and complexity of CAD was determined through angiography and SYNTAX score, respectively. Anthropometric parameters included body mass index (BMI) and blood pressure. The exclusion criteria were patients with thyroid, renal, or hepatic disease, and patients with previous coronary angioplasty or previous revascularization surgery. The estimated glomerular filtration rate (eGFR) was calculated according to CKD-EPI method [27].

Epicardial adipose tissue biopsies were obtained from the atrio-interventricular groove during surgery and placed immediately in tubes with RNaLater™ Stabilization Solution (Life technologies, Carlsbad, CA, USA) and frozen at –20 °C for further analysis.

This study was approved by the Ethics Committee of the National Institute of Cardiology “Ignacio Chávez” with the number 13–818. Participants were informed about the objectives of the study and those who agreed to participate signed a letter of informed consent.

Laboratory assessment
Blood samples were obtained in EDTA tubes after 12 h overnight fasting, centrifuged to 4 °C, the plasma was separated and analyzed. Plasma total cholesterol (TC), triglycerides (TG) and glucose were determined by commercially available enzymatic colorimetric methods (Randox LTD, Crumlin, UK). For lipids associated to HDLs, the phosphotungstic acid-Mg2+ method was used to precipitate apo B-containing lipoproteins before quantifying cholesterol, triglycerides (TG) (Randox, UK), phospholipids (PH) (Wako Chemicals, Richmond, VA) and free cholesterol (FC) (using non-commercially available enzymatic mixtures
recently developed by our group) plasma concentrations. Esterified HDL-cholesterol was calculated as the difference between total cholesterol and free cholesterol concentrations multiplied by a factor of 1.68 to be expressed in milligrams per deciliter (mg/dL). Low-density lipoprotein-cholesterol (LDL-C) was estimated with the formula of Friedewald.

Quantification of mRNA
Total RNA was isolated from tissue samples using the RiboPure™ RNA Purification Kit following the manufacturer’s instructions (Ambion, Life Technologies, Carlsbad, CA, USA). RNA quantity and purity was assessed spectrophotometrically using Nanodrop technology. RNA was reverse transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) in line with the manufacturer’s protocol. qPCR of OPN, ON and OPG mRNA was performed using TaqMan Gene Expression Assays (Invitrogen/Life Technologies, Carlsbad, CA) and compared with the housekeeping gene GAPDH. The primers used were Hs00959010_m1 (OPN); Hs00234160_m1 (ON); Hs00900358_m1 (OPG); and Hs0275899_g1 (GAPDH) all specific for human. Quantification of gene expression was calculated by the standard curve method and normalized to GAPDH.

Isolation of HDL particles and analysis of their structure
HDL were separated by ultracentrifugation and dialyzed against 0.09 M Tris/0.08 M Boric acid/3 mM EDTA buffer, pH 8.4 [28]. The homogeneity and hydrodynamic diameter of HDL subclasses were estimated as previously described [28]. Briefly, HDL samples were separated according to their size by non-denaturing 3% – 30% polyacrylamide gradient gel electrophoresis. Gels were stained for total cholesterol, free cholesterol, phospholipids, and triglycerides using methods recently developed by our group [28]. For the classification of the HDL subclasses, we considered the following size intervals: HDL3c, 7.94–8.45 nm; HDL3b, 8.45–8.98 nm; HDL3a, 8.98–9.94 nm; HDL2a, 9.94–10.58 nm; and HDL2b, 10.58–13.59 nm [28].

Statistical analysis
Normally distributed data, determined with a Kolmogorov–Smirnov test, were expressed as means and standard error (SE). To determine if the contribution of the drug consumption, smoking habit, and the presence of diabetes were statistically significant, we performed a χ² test. Student’s unpaired t-test was used for comparison between groups. Pearson’s correlation coefficients were determined for the expression levels and dimensional variables. A stepwise linear multiple regression model was used to examine the impact of variables on mRNA expression levels. The variables selected for the multiple regression analyses were those with a significant correlation in the bivariate analysis including potential confounding variables to correct the model. The relationship between the presence of atherosclerotic plaque and gene expression was explored using binary logistic regression; the results derived from this analysis were expressed as odds ratio and 95% confidence intervals. All reported P-values were based on two-sided tests. P < 0.05 values were considered as significant. Analyses were performed with the Statistical Package for the Social Sciences version 21.0 (SPSS, Inc., Chicago, IL, USA).

Results
Patient’s anthropometric and biochemical data are shown in Table 1. Mean age and systolic blood pressure were comparable between CAD and control groups, whereas body mass index (BMI) was significantly higher in CAD patients. Drugs intake and smoking habit were similar in both groups (Table 1). Only two individuals in control group reported excessive alcohol intake occasionally. On the basis of the hormone plasma levels and estimated GFR, thyroid and renal function were normal in both groups (Table 1). On the other hand, CAD group had lower plasma concentrations of total cholesterol (30%) and LDL-C (36%), and higher concentrations of glucose than control group.

The lipid profile of the HDLs showed lower plasma concentrations of total cholesterol and esterified cholesterol in the CAD patients compared with the control group (Table 1). These alterations in the lipid profile suggested modifications of HDL subclasses; therefore, we analyzed the lipid composition of these subclasses, and results are shown in the Table 2. The plasma concentrations of total cholesterol and esterified cholesterol of HDL2a, HDL3a, and small HDL3b and 3c were lower in CAD patients than in control group. The concentration of triglycerides was lower in the HDL3b and 3c subclasses (28 and 62%, respectively) in the CAD group whilst the phospholipids and free cholesterol were comparable between both groups (Table 2).

We further calculated the cholesterol-to-phospholipids (TC-to-PH) and triglycerides-to-phospholipids (TG-to-PH) ratios as an approach of the HDLs composition (Table 3). The TC-to-PH ratio from CAD group was significantly lower in the HDL2b, 2a and 3a while the TG-to-PH ratio of the HDL3c was lower compared with the control group. The relative size distribution of HDL subclasses, expressed as percentage of the total HDL protein, was only slightly different between groups; CAD group had a higher proportion of the HDL3b (9%) concomitant with a lower proportion of HDL3c (17%) than control group (Table 1).

The relative mRNA abundance in adipose tissue is shown in Fig. 1. OPN expression was 80% higher in the
between HDL subclasses and gene expression, we performed a correlation analysis; both, the proportion and lipid composition of the HDL subclasses were associated with the expression of these genes (see Additional file 1). Notably, the relative proportion of the HDL3a and 3b subclasses correlated with ON expression ($r = 0.643, p = 0.001; r = 0.484, p = 0.016$, respectively) while HDL3c had a negative correlation with OPG expression ($r = -0.430, p = 0.032$). Plasma concentrations of total cholesterol associated with HDL3a and 3b correlated negatively with OPG expression ($r = -0.567, p = 0.004; r = -0.454, p = 0.026$, respectively).

To identify the potential independent factors that determine the gene expression, we performed a linear multiple regression analysis, considering BMI as covariable; the expression level of each gene was included as dependent variable and the parameters that correlated significantly in the bivariate analysis as independent variables (Additional file 1: Table S1). Lipids associated to total HDLs and those associated to HDL subclasses were included in two different models because of the co-linearity between these parameters. We did not find any association between plasma total HDL-lipids and gene expression (data not shown). Relative size proportion of the HDL3a was a predictor of the ON expression whilst HDL3b was for OPG expression (Table 4). We performed a second linear regression analysis including the cholesterol associated to HDL subpopulations in order to determine which of the HDL subclasses were specifically associated with the gene expression; only HDL3a-TC was associated with OPG expression (Table 4).

We further analyzed whether the gene expression was related with the presence of significant coronary lesions. In the logistic regression analysis, we included the presence of significant coronary lesions as dependent variable and the mRNA expression of the analyzed genes as independent variables. This analysis demonstrated that only ON expression was positively associated with the presence of atherosclerotic plaque (Odds ratio $= 5.625, 95\% \text{ CI} [1.077–29.371], p = 0.041$).

### Discussion

In this study, we demonstrated that the epicardial adipose tissue (EAT) expressed the mRNA of genes related with calcification of the atheroma as OPN, ON and OPG, and that the presence of atherosclerotic plaques was associated with the gene expression of OPN. We also demonstrated that the composition and relative size distribution of the HDL correlated with the expression of the genes analyzed, suggesting an additional function of these lipoproteins.

Patients with aortic valve stenosis without significant coronary lesions were included as non-CAD group. The

| Table 1 Clinical characteristics                                                                 |
|--------------------------------------------------------------------------------------------------|
|                                                                                                  |
| Age (years)                                       Controls (n = 15) | CAD (n = 15) |
| Sex (M/F)                                         58.86 ± 2.19     | 61.58 ± 2.60  |
| BMI (kg/m²)                                       11/4             | 10/5           |
| statin use, n (%)                                 25.69 ± 1.01     | 29.66 ± 1.28*  |
| Anti-hypertensive agents, n (%)                  3 (20%)          | 3 (20%)        |
| Anti-platelet agents, n (%)                      10 (67%)         | 9 (60%)        |
| Anti-diabetic agents, n (%)                      2 (13%)          | 4 (27%)        |
| Organic Nitrates, n (%)                          4 (27%)          | 6 (40%)        |
| Aspirin, n (%)                                    2 (13%)          | 3 (20%)        |
| BMI (kg/m²)                                       122.93 ± 4.26    | 127.50 ± 5.38  |
| SBP (mmHg)                                        73.93 ± 3.79     | 75.33 ± 3.99   |
| Creatinine (mg/dL)                               0.86 ± 0.06      | 0.87 ± 0.07    |
| eGFR (mL/min/1.73 m2)                            60.00 ± 0.01     | 59.80 ± 0.20   |
| TSH (mIU/L)                                       2.42 ± 0.52      | 3.33 ± 0.45    |
| T4 (µg/dL)                                        8.12 ± 0.86      | 8.12 ± 0.45    |
| T3 (ng/mL)                                       1.16 ± 0.04      | 1.14 ± 0.02    |
| Cholesterol (mg/dL)                              147.82 ± 13.28   | 103.08 ± 14.73*|
| Triglycerides (mg/dL)                            99.09 ± 6.49     | 100.40 ± 6.49  |
| Glucose (mg/dL)                                  92.84 ± 2.55     | 106.21 ± 6.19* |
| HDL-cholesterol (mg/dL)                          40.67 ± 3.20     | 31.32 ± 2.17*  |
| Free HDL-cholesterol (mg/dL)                     10.36 ± 1.71     | 11.77 ± 1.04   |
| Estenified HDL-cholesterol (mg/dL)               50.91 ± 5.37     | 32.84 ± 4.54*  |
| HDL- phospholipids (mg/dL)                       29.63 ± 3.13     | 24.78 ± 1.99   |
| LDL-cholesterol (mg/dL)                          70.31 ± 5.04     | 70.41 ± 5.33   |
| HDL 2b (%)                                       98.18 ± 6.65     | 62.51 ± 4.70*  |
| HDL-cholesterol (mg/dL)                          17.59 ± 0.82     | 20.83 ± 1.90   |
| HDL 2a (%)                                       11.40 ± 0.30     | 12.25 ± 0.46   |
| HDL 3a (%)                                       26.82 ± 1.03     | 27.05 ± 0.40   |
| HDL 3b (%)                                       18.69 ± 0.40     | 20.29 ± 0.61*  |
| HDL 3c (%)                                       24.49 ± 1.31     | 20.22 ± 1.44*  |
| SYNTAX score                                     0.00 (0.00–0.00) | 10.00 (2.00–44.00) |

CAD coronary artery disease; BMI body mass index; DM2 type 2 diabetes mellitus; SBP systolic blood pressure; HDL high density lipoprotein; LDL low density lipoprotein; M male; F female; eGFR estimated glomerular filtration rate; TSH thyroid stimulating hormone; T4 thyroxine; T3 triiodothyronine Data are shown as mean ± SE. Student’s t test *p < 0.05 vs. control group.

epicardial adipose tissue from CAD group compared with the control subjects. OPG was expressed twice in the CAD group than in the EAT obtained from the control group (Fig. 1), whereas ON expression was not different between groups.

To explore whether there was a statistical relationship between HDL subclasses and gene expression, we performed
Table 2 Lipid plasma concentrations of HDL subclasses

| Group | Subclasses | HDL 2b | HDL 2a | HDL 3a | HDL 3b | HDL 3c |
|-------|------------|--------|--------|--------|--------|--------|
| TC (mg/dL) | Control | 11.74 ± 1.11 | 5.71 ± 0.44 | 11.19 ± 0.80 | 6.16 ± 0.47 | 6.16 ± 0.42 |
|     | CAD | 9.27 ± 1.12 | 4.26 ± 0.25* | 7.92 ± 0.64* | 4.59 ± 0.35* | 3.43 ± 0.24† |
| FC (mg/dL) | Control | 3.53 ± 0.55 | 1.34 ± 0.19 | 2.64 ± 0.45 | 1.08 ± 0.18 | 1.13 ± 0.16 |
|     | CAD | 4.00 ± 0.34 | 1.60 ± 0.13 | 3.06 ± 0.27 | 1.46 ± 0.12 | 1.14 ± 0.11 |
| EC (mg/dL) | Control | 14.62 ± 1.84 | 7.33 ± 0.72 | 13.73 ± 1.53 | 8.66 ± 0.73 | 8.02 ± 0.57 |
|     | CAD | 8.26 ± 1.85 | 4.47 ± 0.43* | 9.55 ± 1.01* | 5.29 ± 0.59* | 3.84 ± 0.33† |
| TG (mg/dL) | Control | 8.56 ± 1.10 | 4.63 ± 0.56 | 8.40 ± 0.79 | 4.13 ± 0.46 | 4.37 ± 0.45 |
|     | CAD | 10.14 ± 0.97 | 4.20 ± 0.43 | 6.56 ± 0.43 | 2.97 ± 0.21* | 1.66 ± 0.23† |
| PH (mg/dL) | Control | 21.08 ± 1.07 | 9.52 ± 0.54 | 18.20 ± 1.12 | 10.11 ± 0.90 | 10.65 ± 1.52 |
|     | CAD | 25.55 ± 2.00 | 10.42 ± 0.76 | 16.58 ± 1.23 | 8.86 ± 0.66 | 7.18 ± 0.63 |

TC total cholesterol; FC free cholesterol; EC esterified cholesterol; TG triglycerides; PH phospholipids
Data are shown as mean ± SE. n = 15 by group. Student’s t test †p < 0.01, and *p < 0.05 vs. control group

Table 3 Cholesterol-to-phospholipids (TC-to-PH) and triglycerides-to-phospholipids (TG-to-PH) plasma concentration ratios of the different HDL subclasses

| Ratio | Group | Subclasses | HDL 2b | HDL 2a | HDL 3a | HDL 3b | HDL 3c |
|-------|-------|------------|--------|--------|--------|--------|--------|
| TC/PH | Control | 0.55 ± 0.04 | 0.60 ± 0.04 | 0.59 ± 0.03 | 0.57 ± 0.02 | 0.57 ± 0.06 |
|     | CAD | 0.38 ± 0.05* | 0.43 ± 0.03* | 0.49 ± 0.03* | 0.53 ± 0.03 | 0.48 ± 0.05 |
| TG/PH | Control | 0.36 ± 0.05 | 0.42 ± 0.07 | 0.46 ± 0.05 | 0.42 ± 0.05 | 0.41 ± 0.06 |
|     | CAD | 0.41 ± 0.04 | 0.38 ± 0.03 | 0.41 ± 0.03 | 0.35 ± 0.03 | 0.24 ± 0.03* |

Data are shown as mean ± SE. n = 15 by group. Student’s t test *p < 0.05 vs. control group
from CAD patients could be a mechanism to counterbalance the effects pro-inflammatory and pro-calcifying of osteopontin. More studies in vitro and in vivo are needed to evaluate this possibility.

Our results also showed that the gene expression of ON mRNA was expressed at similar levels in both, CAD and control group. The role of osteonectin on the calcification process is controversial [8, 26, 37, 38] and may be determined by the presence of other related proteins; more studies are required to establish whether or not the osteonectin has any contribution in the etiology of the atheroma.

A previous study from our group demonstrated a negative correlation between calcium score and HDL2a, 3a- y 3b–phospholipids [26]. In addition, subjects with high-coronary artery calcium score (CAC) had a lower number of HDL particles than subjects with CAC = 0 [26]. These results suggested that HDL particles regulate the expression of genes encoding for proteins that participate in the formation of the atheroma and its calcification. In order to gain more insight about this possibility, we performed the determination of the composition and size distribution of HDL in both groups of the present study. CAD patients showed lower HDL-cholesterol plasma concentrations than the control group. For a long time, it has been considered to HDL-cholesterol as marker of CAD risk [39, 40]; however, the contribution of the HDL to retard the atherosclerotic process is still controversial. Therefore, it is necessary to determine all lipid components to identify the characteristics that better denote their anti-atherogenic role. In this way, CAD patients had lower concentrations of HDL-EC without significant changes in TG and PH. These differences in plasma concentrations indicate HDL subclasses alterations. For this reason, we further determined HDL subclasses lipid composition.

Esterified cholesterol of the intermediate size HDLs was lower in the CAD group than in controls whereas free cholesterol did not contribute to the changes in plasma levels. In addition, triglycerides plasma concentrations of small HDL particles were lower in the CAD group than in control group, supporting the idea of a significant change of HDLs structure in CAD patients. Therefore, we calculated the TC-to-PH and TG-to-PH ratios; plasma concentrations of phospholipids of HDL subclasses are essential to maintain the structure and size of these lipoproteins; thus, phospholipids of HDL subclasses have been considered as a marker of the number of particles in plasma [41]. In this context, four HDL subclasses were poor in cholesterol while HDL3c had a low content of triglycerides. The observed lipid modifications were accompanied by a slight displacement of HDL subclasses towards large HDLs. Since HDLs structure seems to be associated with the anti-atherosclerotic role of these lipoproteins [41], it is likely that the differences between HDL subclasses in CAD and controls are related with the development of the atheroma in coronary arteries.

Recently, it has been demonstrated that HDL induce the secretion of osteoproterogenin in cultured aortic valve myofibroblasts [20]; the correlations between the calcium score and phospholipids of HDL subclass previously reported [26] are in line with those evidences. Our data demonstrated a positive correlation between the gene expression of OPG and the relative proportion of the HDL3b as well as with HDL-FC of the small particles. Such results further support the possible contribution of these lipoproteins to the calcification process. Moreover, the multivariate analysis showed that for each 1 mg/dL of HDL3a- cholesterol plasma concentration increases, OPG expression decreased 0.137 units. Furthermore, every 1% of increment in the proportion of HDL3a and 3b resulted in an increment of 0.273 and 0.222 times of the ON and OPG expression, respectively. These statistical associations suggest a new role of the HDLs in the progression of atherosclerosis through the regulation of the expression of pro- and anti-calcifying genes in the EAT.

We recognize as an important limitation of the present study that mRNA levels are not always proportional to the amount of the secreted protein; this is only an initial approach to the possible contribution of EAT to the

![Image](image-url)

**Fig. 1** Gene expression for OPN, ON and OPG. Data represent mean ± SE. n = 15 by group. Student’s t test (*p < 0.05). OPN: osteopontin, ON: osteonectin, OPG: osteoprotegerin
development of CAD. The lack of the coronary calcium score is also an important limitation of our study as well as the limited number of patients. Finally, it is necessary to determine if the EAT volume is associated with expression of the genes analyzed.

**Conclusion**

In conclusion, the EAT expresses the mRNA of three genes that have been related with the calcification process, and that such expression is statistically associated with some components of HDL subclasses. These results suggest a contribution of the EAT to the calcification of the atherosclerotic plaque and a new role of the HDLs.

**Additional file**

**Additional file 1: Table S1.** Bivariate correlation analysis between gene expression and subclasses of HDLs. Only statistically significant correlations are shown. TG: triglycerides; TC: total cholesterol; FC: free cholesterol; EC: esterified cholesterol; HDL: high-density lipoprotein (DOCX 17 kb)

**Abbreviations**

CAC: coronary artery calcium; CAD: coronary artery disease; EAT: epicardial adipose tissue; EC: esterified cholesterol; FC: free cholesterol; GAPDH: glyceraldehyde-3-phosphate dehydrogenase gene; ON: osteonectin gene; OPG: osteoprotegerin gene; PP: phospholipids; RANKL: the receptor activator of NF-kB ligand; TC: total cholesterol; TRAIL: TNF-alfa related apoptosis-inducing ligand; VSMC: vascular smooth muscle cells

**Acknowledgments**

None declared.

**Funding**

This study was supported by a CONACyT grant No. 233493. María de Jesús Luna Luna is a doctoral student from Programa de Doctorado en Ciencias Biomédicas de la Universidad Nacional Autónoma de México and received a fellowship from CONACyT, No. 408097.

**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article (and its additional file).

**Authors’ contributions**

Conception and design of the study: ÓP-M, ML-L, NÁ-V, ART-P. Funding ÓP-M. Collection of the samples and generation of data: ML-L, VH-A, JM-R, SC-V, JS-Z, JV-B, CM-S, DC-R, ECT. Analysis, interpretation of data, critical review of the manuscript: ÓP-M, ML-L, GV-A, JS-Z, JV-B. Drafting and revision of the manuscript: ML-L, OP-M, GV-A, ART-P, NÁ-V. All authors read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

This study was approved by the Ethics Committee of the National Institute of Cardiology “Ignacio Chávez”. Participants were informed about the objectives of the study and those who agreed to participate signed a letter of informed consent.

**Consent for publication**

Not Applicable.

**Competing interests**

All authors declare that they have no competing interests.

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Author details**
1 Department of Molecular Biology, Instituto Nacional de Cardiología “Ignacio Chávez”, Juan Badíano 1, Sección XVII, 14080 México City, D.F., Mexico.
2 Department of Echocardiography, Instituto Nacional de Cardiología “Ignacio Chávez”, México City, Mexico.
3 Department of Cardiothoracic Surgery, Instituto Nacional de Cardiología “Ignacio Chávez”, México City, Mexico.
4 Department of Adult Cardiology, Instituto Nacional de Cardiología “Ignacio Chávez”, México City, Mexico.
5 Department of Radiology, Instituto Nacional de Cardiología “Ignacio Chávez”, México City, Mexico.
6 Department of Cardiopulmonary, Instituto Nacional de Cardiología “Ignacio Chávez”, México City, Mexico.
7 Department of Emergency, Instituto Nacional de Cardiología “Ignacio Chávez”, México City, Mexico.
8 Department of Physiology of Nutrition, Instituto Nacional de Ciencias Médicas y Nutrición “Salvador Zubirán”, México City, Mexico.
9 Study Group of Atherosclerosis, Instituto Nacional de Cardiología “Ignacio Chávez”, México City, Mexico.

Received: 28 June 2017 Accepted: 14 August 2017

**References**

1. Iacobellis G, Conrad D, Sharma AM. Epicardial adipose tissue: anatomic, biologic and clinical relationships with the heart. Nat Clin Pract Cardiovasc Med. 2005;2:536–43.
2. Chervin S, Lopaschuk GD, Carvalho E. Cellular cross-talk between epicardial adipose tissue and myocardium in relation to the pathogenesis of cardiovascular disease. Am J Physiol Endocrinol Metab. 2012;303:E937–49.
3. Sacks HS, Fain JN. Human epicardial adipose tissue: a review. Am Heart J. 2007;153:907–17.
4. Lima-Martínez MM, Blandenier C, Iacobellis G. [Epicardial adipose tissue: more than a simple fat deposit?]. Endocrinol Nutr. 2013;60:320–8.
5. Wolak T. Osteopontin - a multi-modal marker and mediator in atherosclerotic vascular disease. Atherosclerosis. 2014;236:32–7.
6. Matsui Y, Rittling SR, Okamoto H, Inobe M, Jin A, Shimizu T, Akino M, Sugawara T, Morimoto J, Kimura C, et al. Osteopontin deficiency attenuates atherosclerosis in female apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2003;23:1029–34.
7. Idoza K, Nishikawa K, Kamezawa Y, Yoshida M, Kusuhara M, Moroi M, Tada N, Ohsuzu F. Osteopontin plays an important role in the development of medial thickening and neointimal formation. Circ Res. 2002;91:77–82.
8. Hirota S, Imakita M, Kohri K, Ito A, Mori E, Adachi S, Kim HM, Kitamura Y, Yutani C, Nomura S. Expression of osteopontin messenger RNA by macrophages in atherosclerotic plaques: A possible association with calcification. Am J Pathol. 1993;143:1003–8.
9. Van Campenhout A, Golledge J. Osteoprotegerin, vascular calcification and atherosclerosis. Atherosclerosis. 2009;204:321–9.
10. Schoppet M, Preissner KT, Hofbauer LC. RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. Arterioscler Thromb Vasc Biol. 2002;22:549–53.
11. Kaden JJ, Bickelhaupt S, Grobholz R, Haase KK, Sarikoc A, Kilic R, Brueckmann M, Lang S, Zahn I, Vahl C, et al. Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulate aortic valve calcification. J Mol Cell Cardiol. 2004;36:57–66.
12. Kanegae Y, Tavares AT, Izpisua Belmonte JC, Verma IM. Role of Rel/NF-kappaB transcription factors during the outgrowth of the vertebrate limb. Nature. 1998;392:11–4.
13. Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dui E, Appelbaum ER, Eichman C, DiPrinzio R, et al. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem. 1998;273:14363–7.
14. Gochuico BR, Zhang J, Ma BY, Marshall-Rothstein A, Fine A. TRAIL expression in vascular smooth muscle. Am J Physiol Lung Cell Mol Physiol. 2002;283:L1045–50.
15. Sato K, Niessner A, Kopecky SL, Frye RL, Goronzy JJ, Weyand CM. TRAIL-expressing T cells induce apoptosis of vascular smooth muscle cells in the atherosclerotic plaque. J Exp Med. 2006;203:239–50.
16. Liaw L, Lindner V, Schwartz SM, Chambers AF, Giachelli CM. Osteopontin and beta 3 integrin are coordinately expressed in regenerating endothelium
in vivo and stimulate Arg-Gly-asp-dependent endothelial migration in vitro. Circ Res. 1995;77:665–72.

17. O’Brien ER, Garvin MR, Stewart DK, Hinchara T, Simpson JB, Schwartz SM, Giachelli CM. Osteopontin is synthesized by macrophage, smooth muscle, and endothelial cells in primary and restenotic human coronary atherosclerotic plaques. Arterioscler Thromb. 1994;14:1648–56.

18. Sattler AM, Schoppet M, Schaefer JR, Hofbauer LC. Novel aspects on RANK ligand and osteoprotegerin in osteoporosis and vascular disease. Calcif Tissue Int. 2004;74:103–6.

19. Hames LW, McCullogh LI, Holley JE, Rawling TJ, Welters HJ, Kos K. A role for SPARC in the moderation of human insulin secretion. PLoS One. 2013;8:e68253.

20. Lommi J, Kovarinen PT, Jauhiainen M, Lee-Rueckert M, Kupari M, Helake S. High-density lipoproteins (HDL) are present in stenotic aortic valves and may interfere with the mechanisms of valvular calcification. Atherosclerosis. 2011;219:538–44.

21. Perez-Mendez O, Pacheco HG, Martinez-Sanchez C, Franco M. HDL-cholesterol in coronary artery disease risk function or structure? Clin Chim Acta. 2014;429:111–22.

22. Williams PT, Krauss RM, Nichols AV, Vanizan KM, Wood PD. Identifying the predominant peak diameter of high-density and low-density lipoproteins by electrophoresis. J Lipid Res. 1990;31:1131–9.

23. Florentin M, Liberopoulos EN, Wierzbicki AS, Mikhailidis DP. Multiple actions of high-density lipoprotein. Curr Opin Cardiol. 2008;23:370–8.

24. Kimura T, Tomura H, Sato K, Matsuoka I, Iim DS, Kubawara A, Mogi C, Itoh H, Kuruse H, et al. Mechanism and role of high density lipoprotein-induced activation of AMP-activated protein kinase in endothelial cells. J Biol Chem. 2010;285:4387–97.

25. Escola-Gil JC, Rotllan N, Julve J, Blanco-Vaca F. In vivo macrophage-specific RCT and antioxidant and antiinflammatory HDL activity measurements: new tools for predicting HDL atheroprotection. Atherosclerosis. 2009;206:321–7.

26. Garcia-Sanchez C, Posadas-Romero C, Posadas-Sanchez R, Carreon-Torres E, Rodriguez-Perez JM, Juarez-Rojas JS, Martinez-Sanchez C, Fraguos JM, Gonzalez-Pacheco H, Vargas-Alarcon G, Perez-Mendez O. Low concentrations of phospholipids and plasma HDL cholesterol subclasses in asymptomatic subjects with high coronary calcium scores. Atherosclerosis. 2015;258:250–5.

27. Levey AS, Stevens LA. Estimating GFR using the CKD epidemiology collaboration (CKD-EPI) creatinine equation: more accurate GFR estimates, lower CKD prevalence estimates, and better risk predictions. Am J Kidney Dis. 2010;55:622–7.

28. Garcia-Sanchez C, Torres-Tamayo M, Juarez-Meavepena M, Lopez-Osorio C, Toledo-Ibelles P, Monter-Garrido M, Cruz-Robles D, Carreon-Torres E, Vargas-Alarcon G, Perez-Mendez O. Lipid plasma concentrations of HDL subclasses determined by enzymatic staining on polyacrylamide electrophoresis gels in children with metabolic syndrome. Clin Chim Acta. 2011;412:292–8.

29. Toro R, Mangas A, Gomez F. [calciﬁed aortic valve disease association with atherosclerosis]. Med Clin (Barc). 2011;136:588–93.

30. Otto CM, Kuusisto J, Reichenbach DD, Gown AM, O’Brien KD. Characterization of the early lesion of ‘degenerative’ valvular aortic stenosis. Histological and immunohistochemical studies. Circulation 1994, 90:844–853.

31. Giachelli CM, Stie tz S. Osteopontin: a versatile regulator of inﬂammation and biomineralization. Matrix Biol. 2000;19:25–38.

32. Jono S, Peinado C, Giachelli CM. Phosphorylation of osteopontin is required for inhibition of vascular smooth muscle cell calcification. J Biol Chem. 2000;275:20197–203.

33. Gerick A, Qin C, Slepak I, Fujimoto Y, Butler WT, Sorensen ES, Boskey AL. Importance of phosphorylation for osteopontin regulation of biomineralization. Calcif Tissue Int. 2005;77:45–59.

34. Luna-Luna M, Medina-Urrutia A, Vargas-Alarcon G, Coss-Rovisosa F, Vargas-Barron J, Perez-Mendez O. Adipose tissue in metabolic syndrome: onset and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at www.biomedcentral.com/submit

BioMed Central