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# Proteomic study of macrophages exposed to oxLDL identifies a CAPG polymorphism associated with carotid atherosclerosis

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ABSTRACT

*Objective:* Macrophages play a key role in the development of atherosclerosis. The objective of this observational study was to characterize the proteome of macrophages to identify proteins implicated in atherosclerosis.
*Methods:* The proteome of macrophage exposed to oxidized low-density lipoprotein (LDL) was studied in a sample of 12 subjects with autosomal dominant hypercholesterolemia and analyzed according to carotid atherosclerosis. Carotid intima-media thickness (IMT), genotyping of the polymorphisms responsible for the amino acid change present in the identified proteins, and an association study was performed in a sample of 320 subjects with autosomal dominant hypercholesterolemia and 145 normolipemic controls.
*Results:* Mass spectroscopy identified two proteins, gelsolin like capping protein (CapG) and glutathione-S-transferase omega 1 (GSTO1), with large variability among subjects which corresponded with two common genetic variants. The rs6886 polymorphism in CAPG was significantly associated with carotid IMT. Carriers of the minor allele in CAPG polymorphism presented less carotid IMT than noncarriers in the hypercholesterolemia group (mean and maximum internal carotid IMT $p = 0.016$ and $p = 0.032$, respectively). This effect was more important in subjects below 50 years old (mean and maximum internal carotid IMT $p < 0.001$).
*Conclusions:* Association analysis revealed rs6886 polymorphism in CAPG to be associated with carotid IMT, suggesting that this polymorphism could modulate macrophages' response to oxidized LDL in subjects with hypercholesterolemia.



## 1. Introduction

Autosomal dominant hypercholesterolemias (ADH) are characterized by a lifelong elevation in the concentration of LDL cholesterol in blood, leading to premature coronary heart disease (CHD), tendon xanthomas (TX) and arcus cornealis [1]. The most common cause of ADH includes defects in the low-density lipoprotein receptor (LDLR) leading to familial hypercholesterolemia [2]. ADH has been used as an LDL dependent atherosclerosis model [2].

In ADH patients, the clinical expression is highly variable in terms of the severity of hypercholesterolemia and CHD even in subjects sharing the same genetic defect [3]. Several genetic and environmental factors explain some of this phenotype variability [4], although most of its causes remain unknown.

Macrophages are the primary cells of the innate immunity and play a key role in all the stages of development of the atherosclerosis plaque [5]. Once in the subendothelial extracellular matrix, LDL is oxidized, a phenomenon promoted by macrophages and endothelial cells. Then, oxidized LDL (oxLDL) is recognized and internalized by macrophage scavenger receptors in an unregulated mode leading to foam-cell formation [6]. Our previous results demonstrated that macrophage inflammatory gene expression is highly variable among individuals when macrophages are exposed to oxLDL and part of this variability could be associated with the macrophage individual response to oxLDL [7].

This study has focused on characterizing the proteome of oxLDL-treated macrophages to identify proteins implicated in the development of CHD in ADH. We hypothesized that macrophages, previously stimulated by oxLDL, express a different proteomic profile associated with a different clinical phenotype.

## 2. Methods

Informed consent was obtained from all subjects. The ethical committee of our institution approved the study.

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### 2.1. Proteomic study

#### 2.1.1. Cases

Twelve subjects with clinical diagnosis of ADH and confirmed causative mutation in the LDLR were selected. When choosing the cases we selected an equal number of subjects with and without atherosclerotic plaque (defined as maximum carotid intima-media thickness (IMT) higher than 1.5 mm, measured in any area of the artery), represented in both sexes. Achilles tendon size was assessed by high-resolution sonography as previously described [8].

#### 2.1.2. Isolation and oxidation of LDL

LDL was isolated from one homozygous familial hypercholesterolemia patient who is regularly treated with dextran sulfate LDL apheresis [7]. The same batch of LDL was used for all the experiments.

#### 2.1.3. Isolation and culture of human monocyte-derived macrophages

Human mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque separating solution (Amersham Biosciences, Uppsala, Sweden) from 40 ml of peripheral blood with 10 U/ml lithium heparin, as described previously [9]. Briefly, mononuclear cells, plated at a density of $1.8 \times 10^6$ cells/ml in five 25-$cm^2$ culture flasks for each subject, were incubated in complete RPMI 1640 with 1% autologous serum for 2 h at 37 °C and 5% $CO_2$. Afterwards, medium was changed and serum content was increased to 10%. 24 h later, monocytes were incubated in macrophage serum-free medium (Invitrogen Life Technologies, Paisley, UK). Monocyte purity was assessed by flow cytometric analysis of CD14 using an Epics XL cytometer (Beckman Coulter, Fullerton, CA, USA) and was routinely found to be >90%. On the ninth day, 50 μg/ml oxLDL was added for 18 h of incubation. The remaining culture was incubated with PBS instead of oxLDL, and was used as a control.

#### 2.1.4. Bidimensional electrophoresis (2DE)

Macrophage lysates were treated with 2D Cleanup kit (Bio-Rad, CA, USA). Samples containing 70 μg protein each were applied by passive rehydration on pH 5–8 IPGs (Bio-Rad, CA, USA). The strips were subsequently equilibrated and loaded onto 12% polyacrylamide gels and separated proteins were detected by BioSafe Coomassie staining. Each gel was scanned and imported in Bio-Rad PDQuest 2D analysis software (Bio-Rad, CA, USA). A qualitative test was applied in order to detect spots that were expressed in one condition but were not expressed in the other. The quantization for a spot to be included in the analysis set was 10 times greater than the minimum detectable spot. The resulting spot sets were visually inspected to verify spot quality.

#### 2.1.5. Mass spectrometry analysis

Protein spots were excised manually and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany) [10]. Data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with a LIFT-MS/MS device [11]. MALDI-MS and MS/MS data were combined through MS BioTools program (Bruker-Daltonics, Bremen, Germany) to search the NCBInr database using Mascot software (Matrix Science, London, UK) [12].

### 2.2. Association study

#### 2.2.1. Cases

320 unrelated Spanish subjects aged 16–82 years with a clinical diagnosis of ADH attending to our institution. Secondary hyperlipidemia and the use of drugs affecting lipid metabolism was ruled out in all subjects. Clinical data, including history of early-onset CHD, demographic and anthropometric characteristics were obtained in each subject.

#### 2.2.2. Controls

This group was composed of 145 unrelated volunteers who underwent a routine medical examination and have been described in part elsewhere [13]. Clinical examination and blood tests were performed in control subjects as in cases. Exclusion criteria was age <16 years old, current acute illness and current use of drugs that modify lipid or glucose metabolism and parental history of dyslipidemia.

#### 2.2.3. Samples

Venous blood samples were collected after a 12-h fast for lipid and lipoprotein measurements. Genomic DNA was isolated using an automated extractor (AutoGenFlex 3000, Qiagen, Hilden, Germany).

#### 2.2.4. Carotid IMT measurements

Carotid IMT was evaluated as previously described [14]. Scans were performed imaging the carotid segments from a fixed lateral angle. The far walls of four carotid segments were visualized: the right and left common-bulb carotid arteries and internal carotid arteries.

#### 2.2.5. CAPG and GSTO1 DNA polymorphisms

Determination of polymorphisms in gelsolin like capping protein (*CAPG*) and glutathione-S-transferase omega 1 (*GSTO1*) genes was performed by PCR followed by restriction enzyme digestion (Hsp92II (Promega, Madison, USA) and Cac8I (New England Biolabs, Ipswich, USA), respectively) and electrophoresis of the digested fragments.

#### 2.2.6. Statistical analyses

Data are presented as mean (±SD) for continuous variables, medians and interquartile ranges for variables with a skewed distribution and as frequencies or percentages for categorical variables. Differences in mean values were assessed using unpaired *t*-test or the Mann–Whitney *U*-test, as appropriate. Categorical variables were compared using chi-square tests. All statistical analyses were performed with SPSS software (version 15.0), with significance set at $p < 0.05$.

## 3. Results

### 3.1. Proteomic study

#### 3.1.1. Cases

12 ADH subjects (5 men and 7 women) were selected. Their main clinical characteristics classified by the presence of atherosclerotic plaque are presented in Table 1.

#### 3.1.2. Bidimensional electrophoresis

After analysis of the gels a region was observed in the macrophage proteome where variability was present: two pairs of spots (named spots A and D and spots B and C) with the same molecular weight but different isoelectric point (Fig. 1). Distribution of the spots of interest in the ADH group is presented in Table 1. Mass spectroscopy analysis concluded that the two pairs of spots were two different proteins, CapG and GSTO1. The difference in the isoelectric point was due to the presence of an amino acid change in each protein: Arg335His in CapG and Ala140Asp in GSTO1 (Fig. 2). To confirm the results obtained with the mass spectroscopy analysis, PCR amplification and enzyme restriction digestion were performed.