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Identification of microRNAs 758 and 33b as potential modulators of ABCA1 expression in human atherosclerotic plaques

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Abstract

Background and aim: Adenosine triphosphate (ATP)-binding cassette (ABC) trans- porters A1 and G1 are the main transporters involved in macrophage cholesterol efflux. The understanding of the molecular mechanism(s) of their regulation in atherosclerosis is crucial f for potential therapeutic approaches. Preclinical studies support a role for microRNAs in the posttranscriptional regulation of these transporters; however, no evidence is still available on human atherosclerosis. Thus, the aim of this study was to investigate the modulation of the ABCA1 and ABCG1 pathway in human atherosclerotic plaques and microRNA involvement in its modulation.

Methods and results: Thirty-one human atherosclerotic plaques were obtained from patients undergoing carotid endarterectomy for high-grade (>70%) vessel stenosis, and divided into normocholesterolemic (*n* Z 15) and hypercholesterolemic groups (*n* Z 16) according to the presence/absence of hypercholesterolemia. Both ABCA1 and ABCG1 messenger RNAs (mRNAs) were significantly upregulated in carotid plaques from hypercholesterolemic patients as as- sessed by real-time polymerase chain reaction (RT-PCR). Despite this result, no difference was found at the protein levels analyzed by Western blot, thus suggesting a strong posttran- scriptional modulation. MicroRNA microarray and subsequent validation by RT-PCR showed a significant upregulation of ABCA1-linked miR-758 and miR-33b in plaques from hypercholes- terolemic patients.

Conclusion: We provide evidence of a strong posttranscriptional regulation of ABCA1 and ABCG1 expression in human atherosclerotic plaques from hypercholesterolemic patients. This effect is potentially due to the concomitant increase of miR-33b and miR-758, two well- established regulators of ABCA1 and ABCG1 expression. The identification of miR-33b and miR-758 as putative key regulators of ABCA1 protein expression within human atherosclerotic plaques provides further data for the realization of new anti-atherosclerotic drugs with specific targets based on anti-miRNA technologies.

Introduction

Atherosclerosis is an inflammatory disease and represents the leading cause of death in Western countries [1]. Cholesterol is involved since the earliest stage of athero-genesis, and strong evidence suggests that a key initiating step is the subendothelial accumulation of Apo-B- containing lipoproteins, such as oxidized low-density lipoproteins (ox-LDLs) [2]. Indeed, ox-LDL exhibits a number of specific biological properties *in vitro* and *in vivo*, including foam cell formation from macrophages, inflammatory response leading to endothelial activation, and migration of inflammatory cells (especially mononuclear phagocytes and T-lymphocytes) [3]. However, cholesterol accumulation within atherosclerotic plaques is the result of the balance between macrophage cholesterol uptake and excretion. Under physiological conditions, macrophages transfer intracellular cholesterol to high-density lipoproteins (HDLs) in the process of reverse cholesterol transport (RCT). The adenosine triphosphate (ATP)-binding cassette A1 (ABCA1) and G1 (ABCG1) are the main transporters responsible for macrophage cholesterol efflux [4]. The expression of both transporters is under the control of the liver X receptors (LXRs), a family of nuclear transcription factors, which act as "cholesterol sensors" promoting cholesterol efflux via the up-regulation of ABCA1 and ABCG1 in states of cholesterol excess [5,6]. In particular, ABCA1 transports intracellular cholesterol to lipid-poor Apo-A1 to generate pre-b-HDL, whereas ABCG1 mediates cholesterol efflux toward mature HDL [4]. ABCA1 deficiency (Tangier disease) or mutation leads to defects in cholesterol efflux and subsequent cholesterol ester accumulation in macrophages, and increases the risk of developing cardiovascular diseases [7]. Furthermore, the combined deficiency of both transporters has been shown to accelerate atherosclerosis by promoting foam cell formation in mice [8].The evidence supports that ABCA1 is expressed in human atherosclerotic plaques and, despite its messenger having been found to be more expressed, conversely, protein levels were reduced in atherosclerotic plaques when compared with healthy vessels [9,10]. Moreover, a difference in ABCA1 modulation has been found between peripheral blood monocytes and atherosclerotic plaques in humans [9], suggesting that ABCA1 modulation might occur at the post-transcriptional level locally within the plaque. However, the intimate mechanism(s) of this modulation has still not been elucidated at this time.

MicroRNAs (miRNAs) are small, endogenous, non-coding regulatory molecules of 20-22 nucleotide length which are able to regulate gene expression at post-transcriptional levels by binding the 3'-untranslated region (UTR) of messenger RNAs (mRNAs) [11]. Their involvement has been widely investigated in many human diseases, ranging from cancer, inflammatory diseases, to cardiovascular diseases [11e15]. Rayner et al. [16] and Najafi-Shoushtari et al. [17] have demonstrated that miR-33 modulates ABCA1, an ABCG1 in both mice and human cells. Interestingly, the inhibition of miR-33a/b raises plasma HDL levels in nonhuman primates and promotes atherosclerosis regression in mice [18,19]. In addition, miR-758 has been implicated in ABCA1 downregulation. Its involvement has been widely demonstrated in human cell lines by using antisense experiments [20]. However, all these evidences derive from in vitro or ex vivo experiments, and from in vivo experiments on animal models, whereas little is known about the modulation of ABCA1 and ABCG1 in human atherosclerotic plaques (particularly in the setting of hypercholesterolemia) and about the role of miRNAs in this context. Recently, we have demonstrated that miRNAs are widely expressed in human atherosclerotic plaques [21,22]; thus, it is conceivable that they could be actively involved in the modulation of cholesterol transporters.

To the best of our knowledge, here we provide the first evidence *in vivo* in humans that hypercholesterolemia is associated with plaque ABCA1 dysregulation and suggest a role for miR-758 and miR-33b as ABCA1 modulators in human atherosclerotic plaques.

Methods

Study population

Thirty-one human atherosclerotic plaques were selected from the MEDICA (MEDIterranean Carotid Atherectomy) biobank of the "G.d'Annunzio" University, Chieti (Italy), a series of >700 atherosclerotic plaques obtained from pa- tients who underwent elective carotid endarterectomy for high-grade (>70%) vessel stenosis. All the patients included in this study were clinically asymptomatic and underwent elective carotid endarterectomy for reducing the long-term risk of stroke. Patients were divided into "normocholesterolemic" (LDL-c < 2.59 mmol/L, n Z 15) and "hypercholesterolemic" groups (LDL-c > 4.14 mmol/L, n Z 16), according to plasma lipid profile values. None of the subjects was diabetic or under any lipid-modifying therapy, in order to reduce potential biases (Table 1). All patients provided their written informed consent. After the surgery, all the plaques were flash-frozen in liquid nitrogen and then stored at 80 °C until the experiments were carried out.

The miRNome cohort

Twenty-two human atherosclerotic plaques, 11 from hy- percholesterolemic and 11 from normocholesterolemic patients, were selected from the above study population in order to realize two pooled RNA samples for miRNome analysis.

Gene expression studies

Atherosclerotic plaques were homogenized using a mortar and pestle. Manual homogenization was preferred to a robotic one because of the plaques' hard composition; anyway, plaque tissue thaw was avoided by keeping it in liquid nitrogen. Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified by spec- trophotometry (GeneQuant pro, GE HealthCare). For gene expression analysis, 1 mg of RNA was reverse-transcribed using a High-Capacity complementary DNA (cDNA) syn- thesis Archive kit (Applied Biosystems, Foster City, CA, USA) and random primers. For miRNA study, 100 ng of total RNA were reverse-transcribed by a MiRCURY LNA Universal RT miRNA polymerase chain reaction (PCR) kit (Exiqon, NASDAQ OMX, Copenhagen, Denmark) according to the manufacturer's instructions. Both gene and miRNAs expressions were measured by real-time PCR (RT-PCR) (ABI7900 HT RealTime PCR System, Applied Biosystem). All TaqMan gene probes were purchased from Applied Bio- system and ribosomal RNA (rRNA) 18S was used as a housekeeping gene. Specific miRNAs primers were pur- chased from Exiqon. MicroRNA-103 and let-7f were selected as the more stable and unaffected miRNAs and were used for data normalization. Gene induction was calculated by the 2^{-DDCt} method.

MicroRNA expression studies

A miRNome analysis was performed on the total RNA extracted from atherosclerotic plaques by using two pre- costumed plates (Human Panel 1&2, Exiqon) containing 742 spotted miRNAs. Briefly, we pooled 300 ng of RNA from each normocholesterolemic or hypercholesterolemic plaque in order to have two final pools, one for each study group. The reverse transcription was performed with a cDNA synthesis kit purchased from Exiqon (MiRcury LNA cDNA synthesis kit), and the obtained cDNA was dispensed on the Human Panel 1 and 2 according to the manufac- turer's instruction. The two plates were calibrated with internal calibrators before normalization. The best

normalizators (hsa-miR-103 and hsa-let-7f) were identified by GeNorm and NormFinder software on these plaques; subsequently, they were validated on all the atheroscle-rotic plaques. Despite being frequently used as a reference gene, U6 was not stably expressed in our samples. For miRNA validation studies on miR-33b and miR-758 (Exi- qon), we used the same cDNA of each plaque originally used for pool preparation, with the addition of the other plaques.

Western blot

After plaque homogenization, proteins were extracted with an extraction buffer composed of radio-immunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail, and phosphatase inhibitor cocktail (Calbiochem, La Jolla, CA). Total proteins were quantified by the Bradford method. Proteins were run under denaturing conditions on a 7% acrylamide gel and then transferred to nitrocellulose. Membranes were then incubated overnight with primary antibodies for ABCA1 (Abcam, Cambridge, UK) and ABCG1 (OriGene Technologies, Rock- ville, MD, USA), and afterward incubated with horseradish peroxidase (HRP)-linked secondary antibodies for the chemiluminescence reaction with ECL (Amersham ECL detection system, GE Healthcare). Protein expression was normalized to b-actin (Sigma Aldrich, Saint Louis, MO, USA) and quantified by densitometry.

Immunohistochemistry

Briefly, tissue cryo-sections of 5-mm thickness were cut and fixed in acetone. After a phosphate buffered saline (PBS) wash, they were incubated for 5 min in a 3% H_2O_2 solution to remove endogenous peroxidases. Slides were firstly incubated 30 min with bovine serum albumin (BSA) 1% solution and then 1 h with the primary antibody (ABCA1 diluted 1:200 in BSA 1%, or CD68 diluted 1:400 in BSA 1%) both after two PBS-Tween 0.1% washes. An HRP polymer conjugate secondary antibody (Invitrogen) was applied for 30 min, with washes in PBS-Tween 0.1% per-formed both before and after the incubation. DAB chromogen solution (Invitrogen) was added for the final step. After a water wash, the slides were counterstained with hematoxylin, washed, dehydrated, and finally mounted for the analysis.

Informatics and statistical analysis

Putative ABCA1-targeting miRNAs were determined by TargetScan (targetscan.org) and Microrna.org prediction software. Statistics were calculated using SPSS v.16.0 (SPSS Inc, Chicago, IL, USA). For gene and miRNA expression data, logarithmic transformation was applied to approximate a Gaussian distribution. The normal distribution of data was tested by the Shapiro & Wilk test. If normal distribution was not confirmed, ManneWhitney *U* test was used to examine differences between groups. Otherwise, Student's *t*-test was used with Welch correction when appropriate. Categorical variables were compared by use of the c^2 test. Correlations were estimated by the Pearson or Spearman correlation test. Differences were considered significant at probability values of <0.05.

Results

ABCA1 mRNA is upregulated in hypercholesterolemic plaques

ABCA1 is one of the most important transmembrane proteins involved in reverse cholesterol efflux with an important atheroprotective role as observed in mice, rats, and swine [8,23]. Because it has been reported that a high- fat diet resulted in ABCA1 modulation together with an increase in atherosclerotic plaque development in animal models [24], we investigated ABCA1 expression in human plaques. We extracted total RNA from human atherosclerotic plaques of patients with and without hypercholesterolemia. RT-PCR data showed a significant increment in both ABCA1 and ABCG1 mRNA expression in hypercholesterolemic plaques as compared to normocholesterolemic ones (2.9- and 3.3-fold increase, respectively), while LXR-a showed a trend in the same direction (Fig. 1A and C). A direct correlation among LXR-a levels and both ABCA1 mRNA (r Z 0.665, p Z 0.004) and ABCG1 mRNA (r Z 0.634, p Z 0.007) was found. Finally, in order to verify the hypothesis of a relationship between hyper-cholesterolemia and plaque ABCA1 induction, we correlated patients' plasma LDL values and plaque ABCA1 mRNA levels. We found a positive correlation between these two parameters (Fig.1D, r Z 0.420, p Z 0.018).

ABCA1 is expressed in macrophages in human atherosclerotic plaques

ABCA1 is mostly expressed by hepatocytes and macrophages. To demonstrate ABCA1 localization in macrophages even in human plaques, we performed immunohistochemistry (IHC) for both ABCA1 and macrophage scavenger receptor CD68. The resulting co-localized signal (Fig. 2A) confirmed macro- phages as the ABCA1 source in both hypercholesterolemic and normocholesterolemic plaques. These data are in agreement with the observation from other research groups; in particular, Chinetti-Gbaguidi et al. have demonstrated that ABCA1 is especially expressed by classic (M1) macrophages in human atherosclerotic plaques [25].ABCA1 protein has a strong post-transcriptional regulation in hypercholesterolemic plaques. Because ABCA1 mRNA resulted to be more expressed in hypercholesterolemic plaques than in normocholesterolemic ones, next we investigated if the protein level showed the same trend. From the same manual homogenization used for miRNome analysis, we took an aliquot of plaque powder in order to extract proteins. Conversely, from mRNA data, Western blot analysis displayed a comparable protein level between the two groups, thus suggesting the existence of a strong post-transcriptional regulation of ABCA1 protein in same trend. From the same manual homogenization used for miRNome analysis, we took an aliquot of plaque powder in order to extract proteins. Conversely, from mRNA data, Western blot analysis displayed a comparable protein level between the two groups, thus suggesting the existence of a strong post-transcriptional regulation of ABCA1 protein in hypercholesterolemic plaques. A similar result was found even for the other transporter ABCG1, which showed the same protein expression level between the two groups (Fig. 2 BeD). Furthermore, we also analyzed the CD68 expression to normalize the ABCA1 protein content on CD68 of each plaque. Interestingly, also after correction for CD68 levels, no significant difference was found in ABCA1 protein expression levels between atherosclerotic plaques from hy- percholesterolemic and control patients (p Z 0.534).

MicroRNA analysis in human atherosclerotic plaques

The comparable level of ABCA1 protein between hyper- cholesterolemic and normocholesterolemic plaques despite the relevant differences in mRNA levels suggested a strong posttranscriptional regulation, especially in hypercholesterolemic patients. Because miRNAs predominantly act as posttranscriptional modulators, we evaluated their expression levels in our study plaques by using miRNome analysis to clarify which miRNAs are modulated by hypercholesterolemia. We ran one pool of cDNA from normocholesterolemic plaques and one pool of cDNA from hypercholesterolemic plaques on two ready-to-use panels (Human Panel I and II), each containing 384 spotted different miRNAs. Once we calibrated and normalized the plates, we excluded from the analysis all miRNAs

showing low expression values (i.e., Ct values >35). After this analysis, we identified several potentially modulated miRNAs, most of them upregulated (Fig. 3).

MicroRNAs 758 and 33b are significantly upregulated in hypercholesterolemic plaques

Recently, other groups showed that miR-33a, miR-33b, and miR-758 downregulate ABCA1 expression in *in vitro* and in animal models [16 e 20]. Matching our results with TargetS- can and microRNA.org databases, we identified miR-33a/b and miR-758 as the putative ABCA1 regulators in human plaques. Notably, these miRNAs have high mirSVR-score values for the interaction with the ABCA1 30-UTR (-1.0397 and -1.0968, respectively). This score could be interpreted as an empirical probability of downregulation, and it is the result of a complex regression model that trains on several features, including base-pairing, secondary structure accessibility, length of 30 UTR region, and others [26].
The subsequent validation study by RT-PCR on each plaque sample showed a strong and significant upregulation of both miR-33b (3.3-fold increase, p Z 0.002) and miR-758 (4.6-fold increase, $p <$ 0.001) in hypercholesterolemic plaques when compared to normocholesterolemic ones (Fig. 4). By contrast, miR-33a expression was not different between normocholesterolemic and hypercholesterolemic plaques, and validation studies showed a non-significant downregulation of this miRNA.

Discussion

RCT is a complex biological process aimed at transferring cholesterol, carried by HDL, from peripheral tissues (including the macrophages in atherosclerotic plaques) to the liver that contributes to its elimination especially through bile acids or feces. The first step in RCT is the cholesterol efflux from macrophages. This step involves several proteins including ABCA1 and ABCG1. In particular, ABCA1 transports intracellular cholesterol to lipid-poor Apo-A1 to generate pre-HDL whereas ABCG1 mediates cholesterol efflux toward further lipidate HDL [4]. The expression of both ABCA1 and ABCG1 is under the control of the LXRs, a family of nuclear transcriptional factors [6]. ABCA1 and ABCG1 are expressed in human athero-sclerotic plaques [9,10,25]. In this study, we found that in hypercholesterolemic patients, despite the high level of ABCA1 mRNA probably due to LXR activation; however, the ABCA1 protein level was low, suggesting the presence of a strong post-transcriptional (or post-translational) mechanism(s) of expression repression. Indeed, in our study, we highlighted how hypercholesterolemia could be interrelated with the increment in ABCA1 mRNA, sug- gesting the existence of an important atheroprotective pathway that may be activated within the plaque in the presence of high tissutal concentration of cholesterol. Unfortunately, we found that this pathway is largely impaired in atherosclerotic plaques from hypercholesterolemic patients, as most of the ABCA1 transcript is not translated into the protein. These data are in agreement with previous reports, which had found an upregulation of ABCA1 mRNA in atherosclerotic plaques when compared with healthy vessels that was not re- flected by a consequent increase in protein level [9,10]. Despite the different experimental setting, these observations also strongly suggest a post-transcriptional modulation of ABCA1 expression occurring within atherosclerotic plaques.
Recent evidences are accumulating about the role of miRNAs in the modulation of both ABCA1 and ABCG1 expression. In particular, Rayner et al. [16] and Najafi- Shoushtari et al. [17] found that miR-33 modulates ABCA1 and ABCG1 in both mice and human cells. Interestingly, inhibition of miR-33 raises plasma HDL in nonhuman primates and promotes atherosclerosis regression in mice [18,19]. In addition, other miRNAs have been involved in ABCA1 modulation, including miR-758, miR-106b, and miR-291-5p [20,27]. Noteworthy, in our study, we have focused on miRNAs with the highest affinity to ABCA1 (as reflected by a high mirSVR-score value) and that had been previously validated as ABCA1 modulators in human macrophages, due to the key role of these cells in atherosclerosis and RCT [16,17,20]. MicroRNAs are greatly expressed in human athero-sclerotic plaques [21,22,28,29]; however, to the best of our knowledge, prior to this study, no evidence was available about the role of miRNAs in the modulation of ABCA1 expression in human atherosclerotic plaques in the setting of hypercholesterolemia. For this purpose, we performed a

miRNome analysis in order to understand how miRNAs are modulated in hypercholesterolemic and normocholester- olemic plaques. We basically analyzed our data and matched them with literature and miRNAs databases. In addition, we focused our attention on miR-ABCA1-binding energy as well in order to look for the best putative miR-NA(s) involved in ABCA1 downregulation. In agreement with both the literature and databases, we found that miR- 33b and 758, the best in animal and *in vitro* characterized miRNAs with respect to ABCA1 downregulation, were both strongly upregulated in the miRNome analysis. Instead, in our study, miR-33a did not show any significant modulation. Validation studies confirmed that these two miRNAs are upregulated in hypercholesterolemic plaques and are associated with reduced translation of ABCA1 protein despite the presence of high levels of mRNA.

Although this study shows some limitations, such as the main associative nature of the study which has not investigated the tiny molecular mechanism(s) of the ob- servations using *in vitro* models, we still believe that our data greatly integrate findings from *in vitro* and in animal studies in the setting of human atherosclerosis, confirming the relevance of data obtained in previous basic science studies, and providing a further step toward translation of miRNA research from an *in vitro* setting to human atherosclerosis.

In fact, despite the evidence that ABCA1 is a key player in cholesterol efflux from macrophages in atherosclerotic plaques, the incomplete clarification of the mechanism involved in its regulation have limited until now the realization of appropriate therapeutic strategies specifically aimed at ABCA1 modulation. MicroRNAs represent a powerful class of modulator of gene expression and their expression could be safely modulated in vivo, also in humans [19,30]. Now, the identification of miR-33b and miR-758 as potential key regulators of ABCA1 protein expression within human atherosclerotic plaques in hy- percholesterolemic patients could provide specific targets for the realization of new atheroprotective and plaque-stabilizing treatment based on selective anti-miRNA technologies.

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Table 1 Study population.

	Hypercholesterolemic Group		Control Group		*p*-value
N	16		15		
Sex (M/F)	12/4		10/5		ns
Age (year)	75.6	8.0	76.5	4.7	ns
BMI (kg/m^2)	25.9	3.0	25.1	3.8	ns
TC (mmol/L)	6.53	0.67	4.40	0.50	<0.001
HDLec (mmol/L)	1.40	0.32	1.38	0.42	ns
Tg (mmol/L)	1.49	0.47	1.73	0.94	ns
LDLec (mmol/L)	4.45	0.53	2.24	0.66	<0.001
Vessel stenosis (%)	79.0	7.8	78.3	8.2	ns
Hypertension	9		13		ns
Diabetes	0		0		ns
History of IHD	4		5		ns
Smokers	2		2		ns

BMI Z body mass index; TC Z total cholesterol; HDL-c Z high-density lipoprotein cholesterol; Tg Z triglycerides; LDL-c Z low-density lipoprotein cholesterol; IHD Z ischemic heart disease.

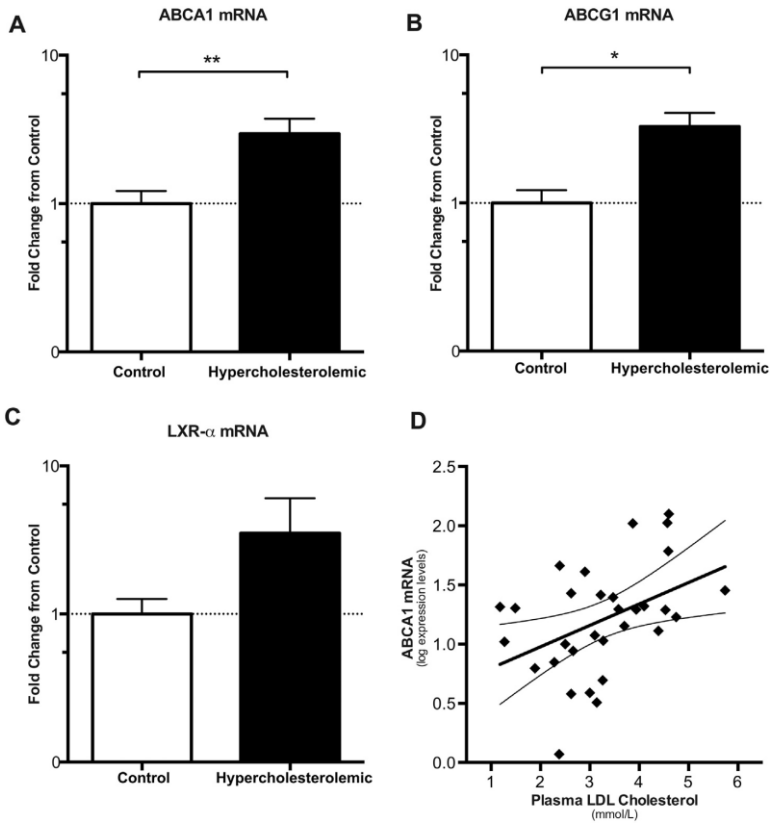

Figure 1 Significant induction of both ABCA1 (A) and ABCG1 (B) at the mRNA level in hypercholesterolemic plaques when compared to normo-cholesterolemic ones. LXR-a also shows a trend toward upregulation in hypercholesterolemic plaques. Data are shown as fold change relative to control group (set as 1.0) (C). Correlation between ABCA1 mRNA and plasma LDL-cholesterol levels. Each point represents one patient (D). *$p < 0.05$; **$p < 0.01$.

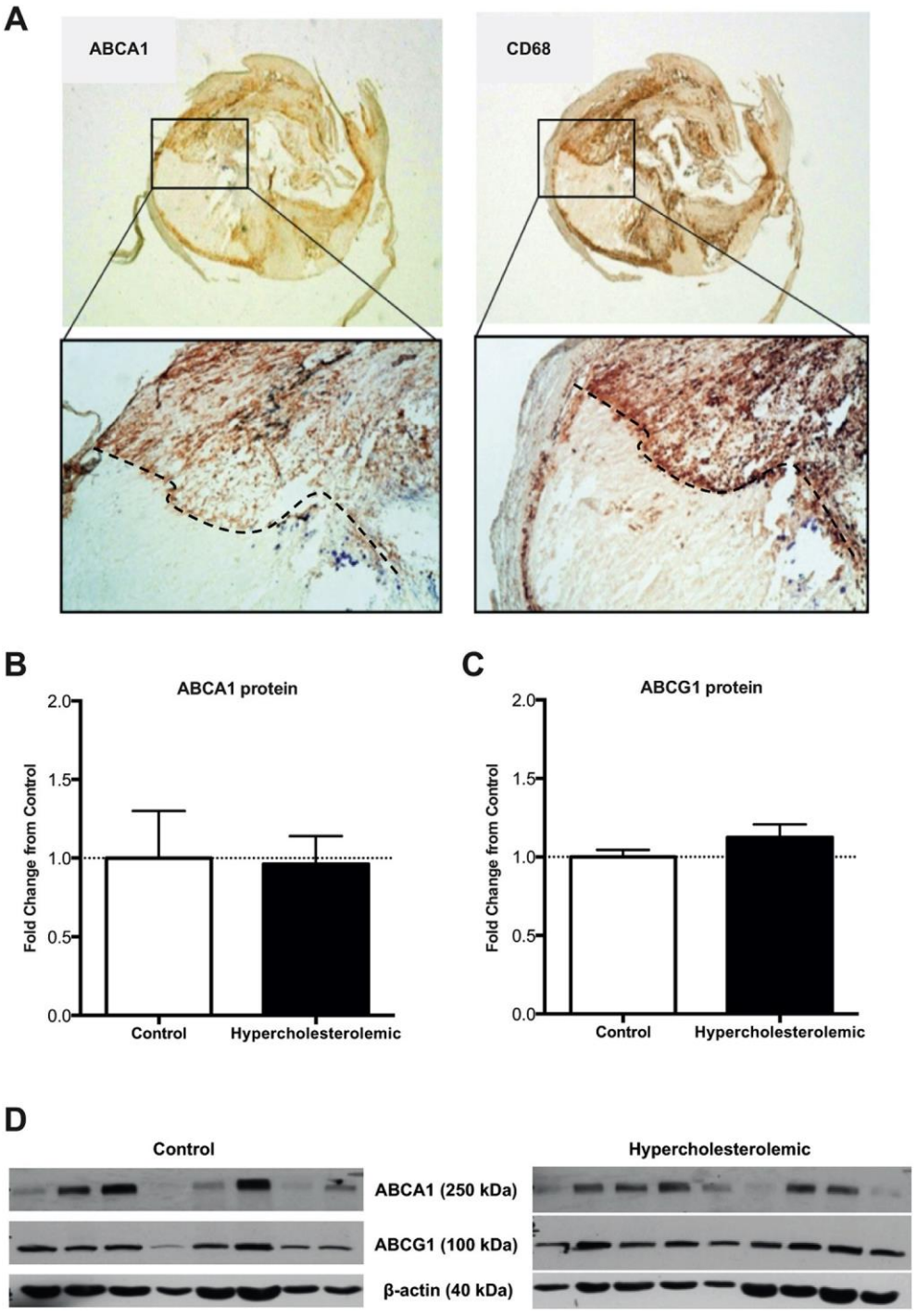

Figure 2 (A) Immunolocalization of ABCA1 protein on macrophages in human atherosclerotic plaques. Positive co-localization of ABCA1 (left panel) antibody and CD68 macrophage scavenger receptor (right panel) is shown. The dashed line represents the lipid core region. (BeD) Western blot analysis for ABCA1 and ABCG1 shows comparable protein levels in atherosclerotic plaques from hypercholesterolemic and normocholesterolemic patients. The protein levels are normalized on b-actin levels. Data are shown as fold change relative to the control group (set as 1.0) (B and C, respectively).

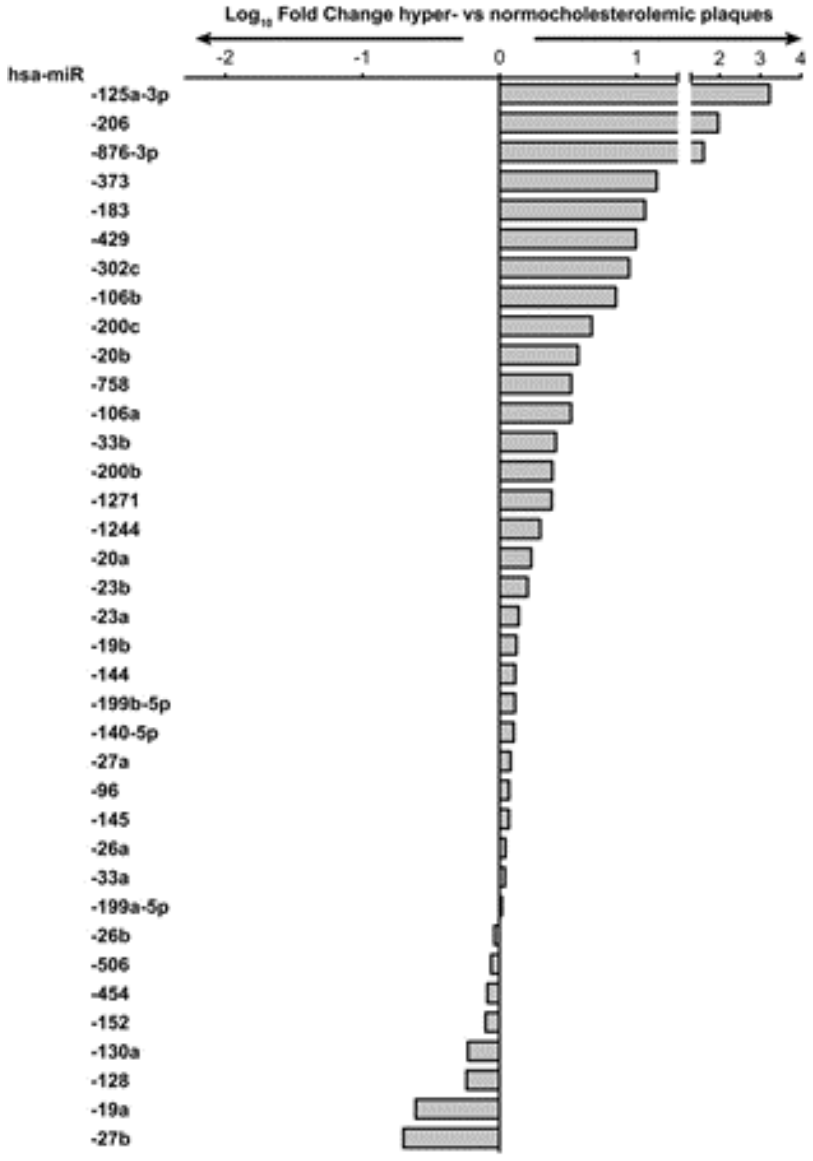

Figure 3 Data obtained by miRNome analysis. Schematic representation of microRNAs in human atherosclerotic plaques as modulated by hypercholesterolemic status. *X*-axis indicates fold changes in the expression levels of each microRNA on a $\log_{10}$ scale.

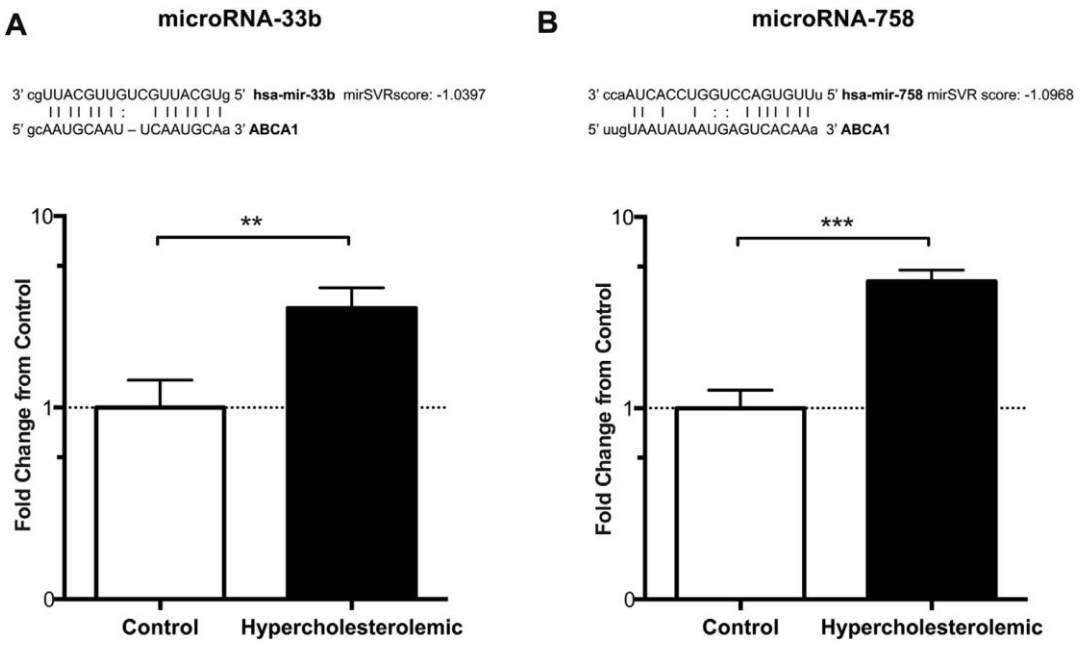

Figure 4 MicroRNA 33b (A) and 758 (B) are upregulated in hypercholesterolemic plaques (*n* Z 16) when compared to normocholesterolemic ones (*n* Z 15). MicroRNA expression was assessed by quantitative real-time PCR. Data are shown as fold change relative to the control group (set as 1.0). Both miRNAs show a remarkable mirSVR score as shown by the microRNA.org website.