

## Research Article

# CD36 Is Significantly Correlated with Adipophilin in Human Carotid Lesions and Inversely Correlated with Plasma ApoAI

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OxLDL uptake and cholesterol efflux inhibition in macrophages play a key role in atherosclerotic plaque formation, rupture, and thrombotic ischemia. This study investigates genes implicated in OxLDL uptake (CD36, SRA), cholesterol efflux inhibition (adipophilin, ADFP), and inflammatory recruitments of leukocytes (IL-8) in plaque lesion areas (PLAs) compared to nonplaque lesion areas (NPLAs) in human carotid endarterectomy specimens. Gene and protein expressions were assayed using quantitative PCR and quantitative immunohistochemistry. Pearson tests were used to investigate potential correlation between (a) different gene expressions and (b) gene expression and patient's plasma constituents. CD36, SRA, ADFP, and IL-8 were shown to be significantly more expressed in PLA compared to NPLA. In PLA, a significant correlation was observed between CD36, SRA, ADFP, and IL-8 mRNA levels. Moreover, CD36 expression level was significantly inversely correlated to plasma marker ApoAI. The above investigated genes/proteins may play a key role in the maturation of atherosclerotic lesions.

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## 1. INTRODUCTION

Macrophage uptake of oxidised low density lipoprotein (OxLDL) and the concomitant reduction in cholesterol efflux are thought to play a critical role in atherosclerotic plaque formation and subsequent rupture. A number of macrophage OxLDL receptors have been identified (e.g., CD36, SRBI, SRA) [1, 2] but two in particular, CD36 and SRA, have been shown to be responsible for up to 90% of OxLDL uptake and degradation by macrophages [3]. A double knockout mouse model of atherosclerosis (ApoE<sup>-/-</sup>, CD36<sup>-/-</sup>) has many fewer and smaller lesions compared to ApoE<sup>-/-</sup> animals that express CD36, even after long-term feeding with a western diet, [4, 5] which indicates that the CD36 scavenger receptor is implicated in the generation of

advanced plaques. This is, however, at variance with the finding that similar double knockout mice have a comparable, or even increased, atherosclerotic lesions in the region of the aortic valve [6] as the ApoE<sup>-/-</sup> mice that have intact CD36, indicating that uptake and endocytosis of OxLDL seem to be independent of CD36 [6]. Different possibilities have been recently reviewed to explain such discrepancies [7, 8]. Furthermore, results from ApoE<sup>-/-</sup> mice have to be interpreted cautiously, as the lesions are different from those observed in humans, often with none of the clinical end points, such as thrombotic ischaemia, resulting in myocardial infarction or stroke [9]. The present work has attempted to overcome this limitation by examining human carotid endarterectomy specimens. Indeed, very few studies have investigated the expression of CD36 in human vascular lesions [10].

Adipophilin (ADFP), a cellular indicator of the amount of stored lipids, is present in a very large number of tissues [11]. Increased expression of ADFP in THP-1 monocytic cell line has been observed to induce cholesterol storage and reduction of cholesterol efflux [12]. Moreover, its expression in macrophages has been correlated with the presence of red blood cells and cholesterol crystals in vascular lesions [13]. Finally, ADFP may play a role in the differentiation of macrophages into foam cells [14].

High density lipoproteins (HDLs) are implicated in the transport of cholesterol to the liver. It is now well established that there is an inverse relationship between the risk of developing cardiovascular diseases and the concentration of HDL. However, the protective effect of HDL is not fully understood. APOAI, the main apolipoprotein of HDL, plays a critical role in cholesterol efflux from macrophages and foam cells present in the vessel wall [15].

This study investigates genes/proteins implicated in OxLDL uptake (CD36, SRA), inhibition of cholesterol efflux (ADFP), and inflammatory recruitments of leukocytes (IL-8) in plaque lesion areas (PLAs) compared to nonplaque lesion areas (NPLAs) in human carotid endarterectomy samples. A substantial difference in mRNA and protein expressions between PLA and NPLA was observed for CD36, ADFP, SRA, and IL-8. In addition, a significant correlation was obtained between CD36 expression and ADFP, SRA and IL-8 in PLA. Moreover, a significant inverse correlation was observed between CD36 gene expression level in PLA and plasma marker ApoAI.

## 2. MATERIALS AND METHODS

### 2.1. Human tissue samples

Ninety patients, subjected to endarterectomy (carotid stenosis >70%) at 3 different hospitals (Red Cross Hospital in Athens (Greece), University Medical Centre in Utrecht (The Netherlands), and Bichat hospital in Paris (France)), were enrolled in this study. After removal, endarterectomy samples were immediately rinsed in RNase-free sterile PBS, submerged in RNALater (Ambion, Tex, USA), and stored at  $-80^{\circ}\text{C}$ . Ethical committees approved the study, and patients were fully informed and gave their written consent prior to enrolment. Information concerning the patients is summarised in Table 1.

### 2.2. Endarterectomy handling

The lesions were classified according to the American Heart Association (AHA) criteria [16] by expert pathologists (Table 1). A transverse (perpendicular to the lumen) section of the internal carotid artery of 5–10  $\mu\text{m}$  thickness was taken in the plaque lesion area (PLA) above the bifurcation point (Figure 1). When available, a macroscopically nonplaque lesion area (NPLA) adjacent to the atheromatous area, removed as a part of the normal surgical procedure, was used as an internal control. The PLA and NPLA sections were split horizontally giving 2 identical pieces of the same area being used, respectively, for immunohistochemistry and for

TABLE 1: Patient phenotypes.

|   | <i>n</i> = 90   |
|---|---|
| Age                                     | 66.5 $\pm$ 8.7  |
| Male/Female                             | 76%/24%   |
| Smokers                                 | 64%   |
| Symptoms                                | 56%   |
| Diabetes mellitus                       | 26.8%   |
| Hypertension                            | 84%   |
| Total cholesterol (mM)                  | 4.8 $\pm$ 0.9   |
| HDL (mM)                                | 1.01 $\pm$ 0.19   |
| LDL (mM)                                | 3.1 $\pm$ 0.8   |
| Triglycerides (mM)                      | 3.9 $\pm$ 2.3   |
| Hematocrit (%)                          | 40 $\pm$ 4.7  |
| White blood cells (*10 <sup>9</sup> /L) | 7.3 $\pm$ 1.4   |
| Platelets (*10 <sup>9</sup> /L)         | 218(115–360)  |
| Glucose (mg/dL)                         | 93 $\pm$ 27.6   |
| Creatinine (mg/dL)                      | 0.9   |
| beta-blockers                           | 32.8%   |
| calcium antagonists                     | 30.1%   |
| ACE inhibitors                          | 42.5%   |
| Diuretics                               | 29%   |
| ARB                                     | 26%   |
| Statins                                 | 49%   |
| Aspirin                                 | 47.9%   |
| Clopidogrel                             | 46.2%   |
| Insulin                                 | 3%  |
| Diabetes mellitus pills                 | 21.9%   |
| ApoAI (mg/dL)                           | 115 $\pm$ 18  |
| ApoB (mg/dL)                            | 92 $\pm$ 23   |
| Lp(a)                                   | 20 $\pm$ 48   |
| Histological grade (AHA)                | 65.7% grade 6<br>28.6% grade 5<br>4.3 grade 4<br>1.4% grade 3 |

quantitative PCR (Q-PCR) (Figure 1). Fibroatheroma (type V) or complicated (type VI) lesions, as described in the AHA criteria constituted the investigated PLA. Both pieces were washed twice in cold RNase-free sterile PBS to remove the RNALater and either included into OCT or immersed into RLT lysis buffer (Qiagen, Calif, USA) to protect the RNA and stored at  $-80^{\circ}\text{C}$  until further use. For all the samples included into OCT, tissue sections were cut at 8  $\mu\text{m}$  thickness, dried, and fixed in acetone. Plaque morphology was also evaluated by staining the tissues with hematoxylin and eosin.

### 2.3. THP-1 cells culturing and stimulation

Human monocytic leukaemia cell line (THP-1/European collection of cell cultures-ECACC no. 88081201) was cultured in RPMI 1640 (Sigma, Mo, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Sigma), 1% glutamine (Sigma), 100  $\mu\text{g}/\text{mL}$  streptomycin (Sigma) in a humid incubator with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . THP-1 cells,

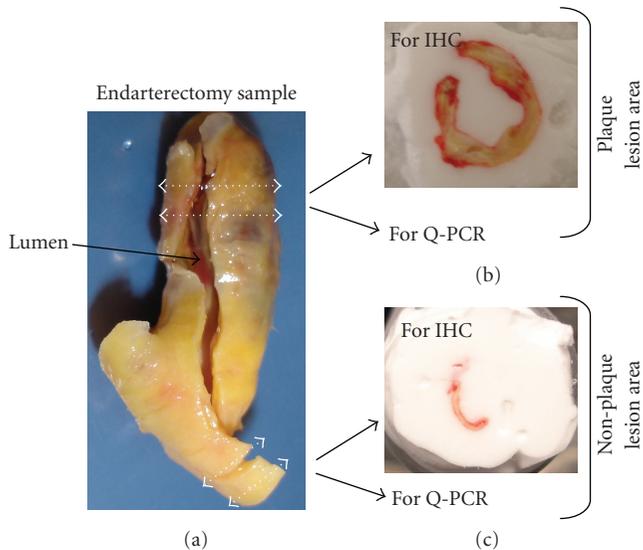


FIGURE 1: (a) Typical macroscopic pictures of a carotid endarterectomy specimen. (b) A transverse (perpendicular to the lumen) section of the internal carotid artery was taken in the plaque lesion area (PLA) above the bifurcation point. (c) A macroscopically nonplaque area (NPLA) was used as an internal control. The PLA and the NPLA sections were split horizontally giving 2 identical pieces that were used, respectively, for immunohistochemistry and for quantitative PCR.

cultured in 6-well plates at a density  $1.2 \times 10^6$  cells/well, were first treated with  $0.2 \mu\text{M}$  phorbol 12-myristate 13-acetate (PMA, Sigma) during 24 hours in order to induce the differentiation of monocytes into macrophages, then washed with sterile PBS and stimulated with  $100 \mu\text{g}/\text{mL}$  oxidised low-density lipoproteins (OxLDL, Intracell, USA) during 24 hours. Cell viability was evaluated using Trypan blue counting to be more than 95% for each assay. Stimulation assays were performed in duplicate and repeated 3 times.

#### 2.4. RNA extraction

**THP-1 cells:** total RNA extractions were performed using the RNeasy Mini Kit (Qiagen) following manufacturer's instructions. RNA quality and quantity were monitored using a Nanodrop spectrophotometer (Nanodrop Technologies, Del, USA) and an RNA 6000 NanoLabChip kit on a Bioanalyser (Agilent Technologies, Calif, USA).

**Tissue samples:** 10 to 30 mg of tissue were used for RNA extraction depending on the tissue size available. Tissues were first cut into very small pieces and homogenised with  $350 \mu\text{L}$  of RLT lysis buffer (Qiagen) using a Polytron ( $2 \times 30$  s on ice). After 10 minutes at  $55^\circ\text{C}$  proteinase K step ( $0.22 \text{ g}/\text{L}^{-1}$ ), the tissue lysate was centrifuged 3 minutes at  $10,000 \text{ g}$  and RNA extraction was performed on the supernatant following manufacturer's instructions using RNeasy Mini Kit (Qiagen). RNA quality and quantity were monitored using RNA 6000 PicoLabChip/NanoLabChip kit on a Bioanalyser (Agilent) and using the Nanodrop spectrophotometer (Nanodrop Technologies). Total RNA

extracted from highly calcified lesions was poor and these were removed from the cohort.

#### 2.5. Retrotranscription (RT) and real-time quantitative PCR (Q-PCR)

Only total RNA extracts presenting good integrity on Bioanalyser profiles were retrotranscribed ( $1 \mu\text{g}$  for THP-1 cells or  $200 \text{ ng}$  for tissue samples) with superscript III reverse transcriptase (Invitrogen) following manufacturer's instructions.

Q-PCR assays were carried out using the 7900 HT Sequence Detector System (Applied Biosystems/Ambion, (AB)). Five microlitres of 10X RT product were amplified using 1X of Universal PCR Master Mix (AB), 1X assay-on-demand (containing specific primers and labelled probe, AB) in a final volume of  $25 \mu\text{L}$ . Q-PCR amplification was performed in duplicate. 18S rRNA (AB) amplification was used for normalisation. Each gene was assayed individually.

#### 2.6. Immunohistochemistry and quantitative image analysis

Serial sections of endarterectomy samples (NPLA and PLA) were first immunostained with an anti-CD68 (1/500, Dako, Glostrup, Denmark), an anti- $\alpha$  actin (1/50, Dako), and an anti-CD31 (1/20, Dako) in order to localise macrophages, smooth muscle cells (SMCs), and endothelial cells, respectively. Then sections were incubated with either an anti-CD36 monoclonal antibody (1/25, BioCytex, Marseille, France), an anti-SRA (1/100, R&D Systems, Minn, USA), an anti-ADFP (1/200, Chemicon, Millipore, Billerica, USA), or the corresponding IgG isotype. Staining was visualised using the Envision kit (Dako). Slides were then counterstained with hematoxylin and mounted in DPX (Sigma). Staining of CD36, SRA, and ADFP was quantitatively analysed by the use of a colour image analyser (Sight System, Newbury, Berks, UK; Software by Foster-Finley Associates, Newcastle, UK) detecting the peroxidase reaction product by measurement of pixels in terms of hue, saturation, and intensity values. By this method, the percentage of the stained tissue was measured. For each tissue, the entire section was analysed and the intensity staining obtained was normalised via the surface of the tissue analysed [17, 18].

#### 2.7. Western-blot

Proteins were extracted using 5%-tritonX-100 lysis buffer. Twenty micrograms of total proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with an anti-CD36 monoclonal antibody (Clone 10/5) (BioCytex), or with an anti-ADFP polyclonal antibody (AB9102) (Chemicon) at dilutions 1/100 and 1/2000, respectively. Detection was performed using ECL western blotting detection reagents (Amersham, Buckinghamshire, UK) and exposure to Kodak Biomax XAR films (Sigma). Signals were analysed and compared using GelDoc software (BioRad Laboratories, Calif, USA).

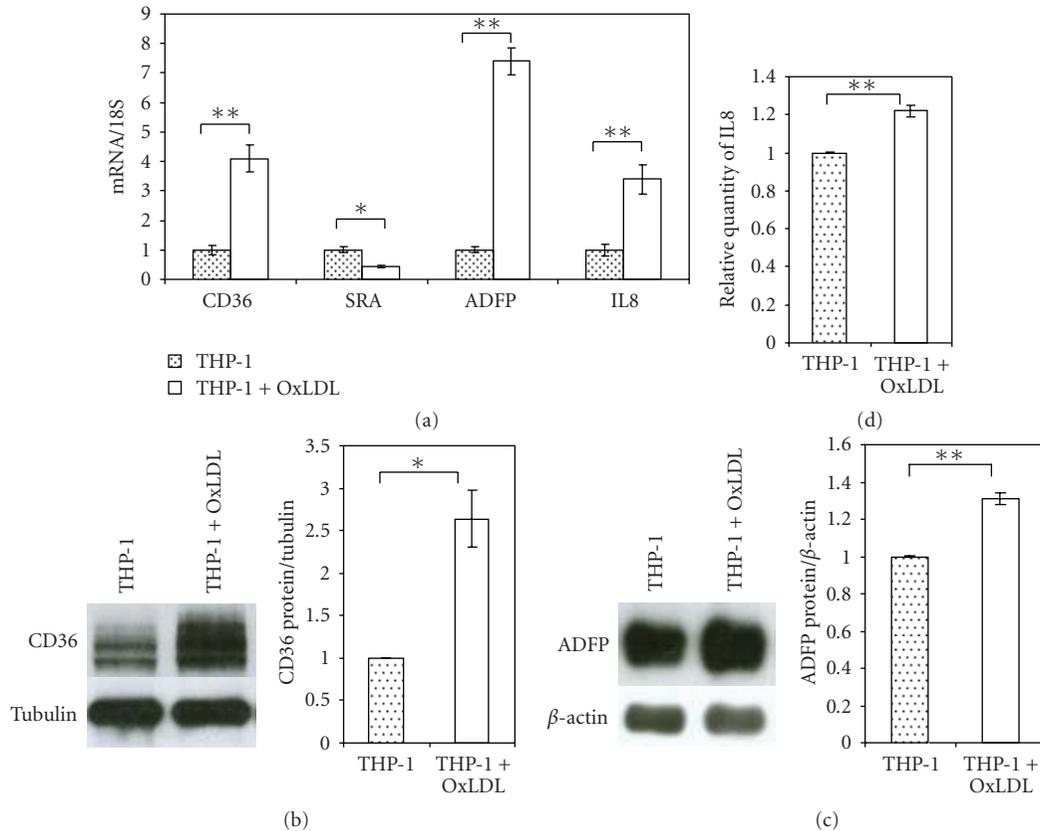


FIGURE 2: Comparison of gene/protein expression patterns between OxLDL-treated (100 µg/mL, for 24 hours) and PBS-treated PMA-differentiated THP-1 cells. (a) Real-time quantitative PCR showing gene expression levels for CD36, SRA, adipophilin (ADFP), and IL-8. (b), (c) Western-blot analysis of, respectively, CD36 and ADFP protein expression levels. (d) IL-8 protein levels in THP-1 culture supernatants assayed by ELISA. Treated and untreated cells are, respectively, represented by plain and dotted bars. Bars represent the means  $\pm$  SE for 3 different experiments performed in duplicate. Wilcoxon matched pairs test was used to determine significance. \*\* $P \leq .01$ , \* $P \leq .05$ .

## 2.8. ELISA

IL-8 levels were measured in cell supernatants in duplicate using an IL-8 ELISA Kit (Quantikine from R&D Systems) according to the supplier's instructions.

## 2.9. Statistical analysis

Statistical analyses were performed using Wilcoxon matched pairs tests (ranks test) and Mann-Whitney tests (Statistica software, Statsoft). Online software ([http://www.fon.hum.uva.nl/Service/Statistics/Correlation\\_coefficient.html](http://www.fon.hum.uva.nl/Service/Statistics/Correlation_coefficient.html)) was used to conduct Pearson's correlation tests. In all cases, a value of  $P \leq .05$  was regarded as significant.

## 3. RESULTS

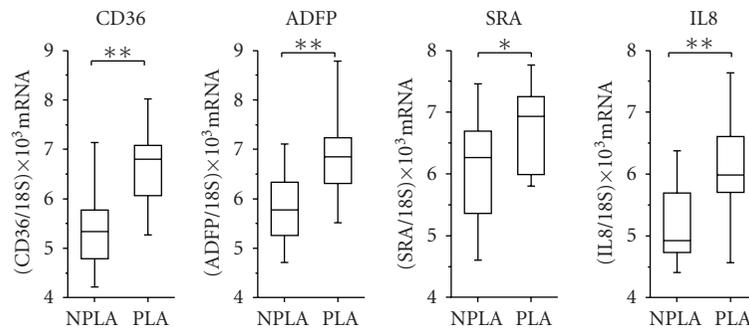
### 3.1. OxLDL stimulation in PMA-differentiated THP-1 macrophages

An in vitro approach was first used to investigate the gene and protein expressions, under our laboratory conditions, of 4 OxLDL-targeted genes (CD36, SRA, ADFP, IL-8) prior to starting the work on endarterectomy tissues. Twenty

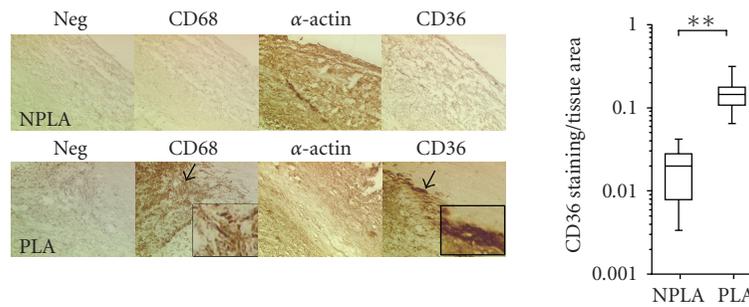
four hours of OxLDL treatment (100 µg/mL) of PMA-differentiated THP-1 macrophages, compared to cells incubated with PBS only, induced a significant increase of CD36 (4.1  $\pm$  1.1-fold), IL-8 (3.4  $\pm$  0.9-fold), and ADFP (7.4  $\pm$  1.4-fold) gene expressions,  $P < .05$  (Figure 2(a)). In contrast, a decrease of SRA gene expression (0.5  $\pm$  0.1-fold) was observed in these cells after OxLDL treatment (100 µg/mL)  $P < .05$ . Western-blot analysis showed that CD36 and ADFP protein levels were also increased respectively by 132% ( $\pm$ 30%) ( $P = .05$ ) and by 31% ( $\pm$ 3%,  $P = .01$ ) after the OxLDL treatment (Figures 2(b) and 2(c)). IL-8 increased by 22% ( $\pm$ 1.1%,  $P = .005$ ) in cell supernatant using ELISA assays (Figure 2(d)).

### 3.2. Comparison of gene and protein expressions in PLA versus NPLA

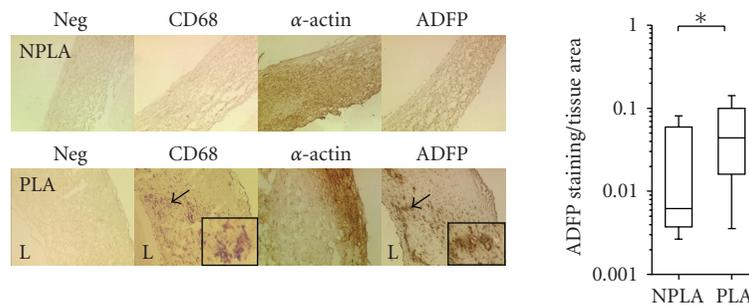
Gene and protein expressions (CD36, SRA, ADFP, and IL-8) at sites of PLA were compared to NPLA in endarterectomy samples. CD36 (6.80 versus 5.41,  $P = .0045$ ), ADFP (6.85 versus 5.78,  $P = .0017$ ), SRA (6.93 versus 6.27,  $P = .23$ ), and IL-8 (5.98 versus 4.93,  $P = .0065$ ) genes were shown to be significantly more expressed in PLA compared to NPLA in the 15 patient's samples investigated (Figure 3(a)). Furthermore, PLA showed significantly higher CD36



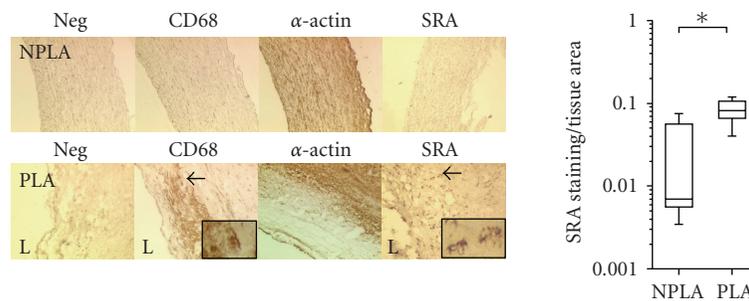
(a)



(b)



(c)



(d)

FIGURE 3: Gene and protein expression levels in carotid endarterectomy PLAs (plaque lesion areas) and NPLAs (nonplaque lesion areas). (a) Real-time quantitative PCR showing gene expression levels for CD36, adipophilin (ADFP), SRA, and IL-8 in carotid endarterectomy PLA compared to NPLA. (b) Staining of negative isotype, CD68,  $\alpha$ -actin, and CD36 proteins in PLA and NPLA and quantitative immunohistochemistry for CD36 protein expression in PLA versus NPLA. (c) Staining of negative isotype, CD68,  $\alpha$ -actin, and ADFP proteins in PLA and NPLA and quantitative immunohistochemistry for ADFP protein expression in PLA versus NPLA. (d) Staining of negative isotype, CD68,  $\alpha$ -actin, and SRA proteins in PLA and NPLA and quantitative immunohistochemistry for SRA protein expression in PLA versus NPLA. Wilcoxon matched pairs test was used to determine significance.  $**P \leq .001$ ,  $*P \leq .05$ ; L: lumen; magnification: 10X.

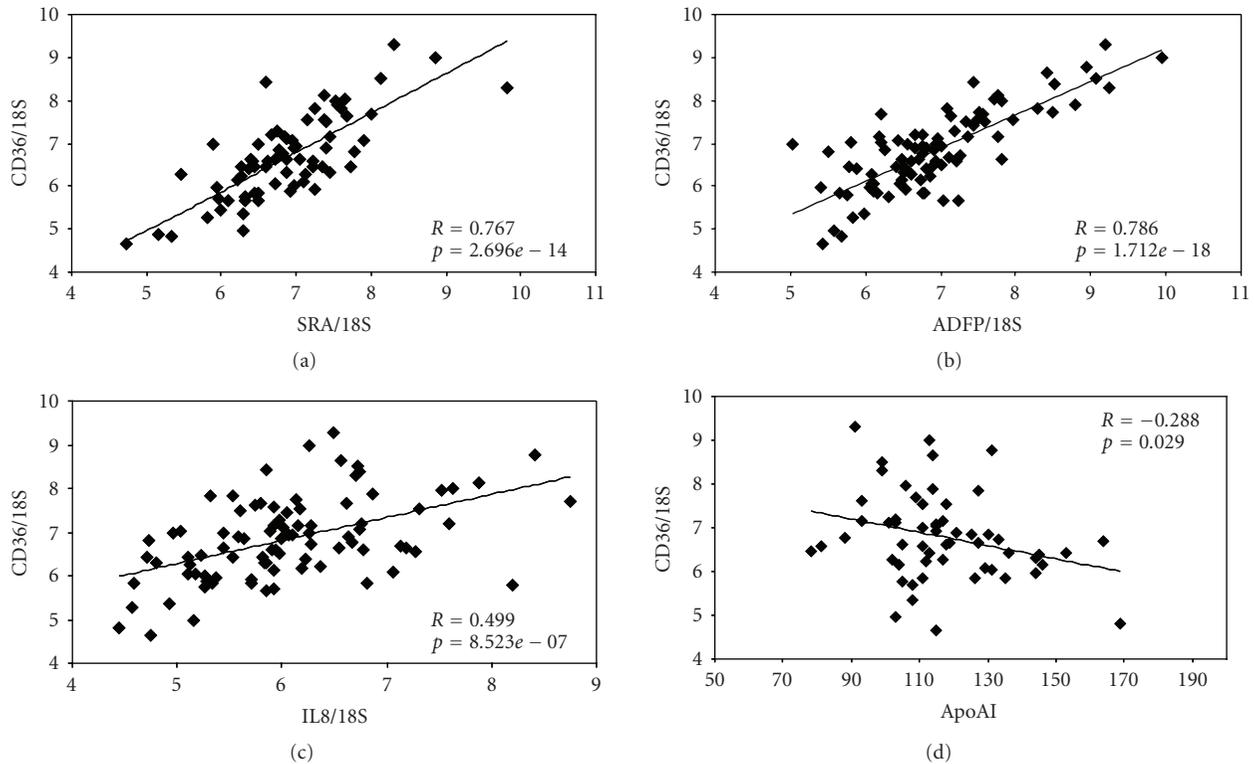


FIGURE 4: Correlation of CD36 with (a) SRA ( $n = 72$ ), (b) adipophilin ( $n = 88$ ), or (c) IL-8 ( $n = 88$ ) gene expression in plaque lesion area (PLA). (d) Correlation of CD36 gene expression in plaque lesion areas (PLAs) with plasma marker ApoAI level in 57 patients. Pearson's correlation statistical test was used.

(0.15 versus 0.019,  $P = .017$ ), ADFP (0.051 versus 0.026,  $P = .049$ ), and SRA (0.081 versus 0.027,  $P = .027$ ) protein levels compared to NPLA using quantitative immunostaining techniques in the 8 patient's samples investigated (Figures 3(b), 3(c), and 3(d)).

### 3.3. Correlation of CD36 with SRA or ADFP in carotid endarterectomy specimens

In PLA samples, a significant correlation was observed between CD36 and SRA mRNA expression levels ( $n = 72$ ,  $R = .767$ ,  $P < .01$ ). Interestingly, a significant correlation was also observed between CD36 and ADFP ( $n = 88$ ,  $R = .786$ ,  $P < .01$ ) and between CD36 and IL-8 ( $n = 88$ ,  $R = .499$ ,  $P < .01$ ) mRNA expression levels (Figure 4).

### 3.4. Correlation between CD36 with ApoAI and HDL

A significant inverse correlation was observed between CD36 gene expression level in PLA samples ( $n = 57$ ) and plasma marker ApoAI ( $R = .288$ ,  $P = .029$ ) (Figure 4(d)). Furthermore, an inverse correlation was also found between CD36 gene expression and HDL plasma level, however it was not statistically significant ( $R = .207$ ,  $P = .1$ ). No correlation was observed between CD36 and total cholesterol, LDL, triglycerides, plasma ApoB, medication, smoking status or gender (data not shown).

## 4. DISCUSSION

In this study, a substantial difference in mRNA and protein expressions was observed for CD36 and molecules implicated in lipid metabolism (ADFP, SRA) and inflammation (IL-8) between plaque lesion area (PLA) and nonplaque lesion area (NPLA) in human carotid endarterectomies. Moreover, results obtained for an in vitro macrophage model (PMA-treated THP-1 cells) stimulated by OxLDL show a trend, with the exception of SRA, that is similar to those obtained in PLA. In addition, a significant correlation was obtained between CD36 expression and ADFP, SRA, and IL-8 in PLA. Furthermore, a significant inverse correlation was observed between CD36 gene expression level in PLA and plasma marker ApoAI.

CD36, ADFP, and SRA are strongly correlated in this study in PLA and are potentially proatherogenic proteins. Indeed, resident vascular macrophages have scavenger receptors CD36 and SRA that play a key role in the uptake of OxLDL. Moreover, ADFP in these very same macrophages is involved in lipid accumulation by inhibiting efflux [12]. It has also been described that the ABCA1 transporter protein expression, involved in the cholesterol transport from cells to apolipoproteins in plasma, was significantly reduced in carotid lesions compared with controls tissues [19]. The uptake of OxLDL upregulates scavenger receptors and the adipophilin expression and ensures the formation of foam cells and the perpetuation of lesions. An inflammatory

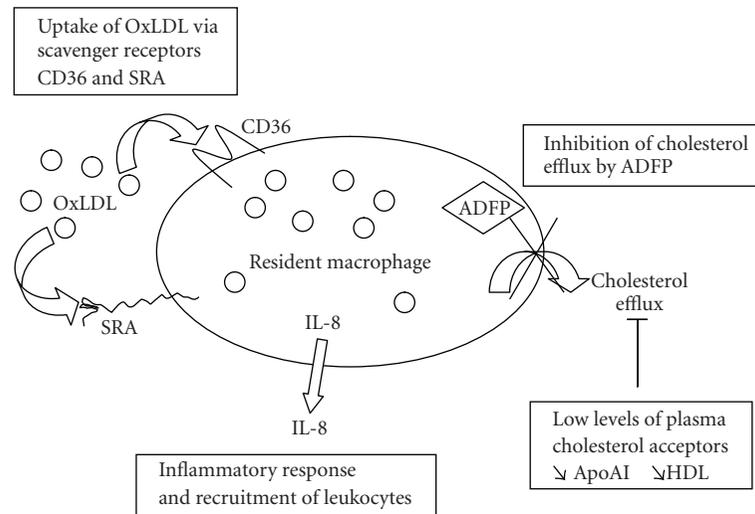


FIGURE 5: Hypothetical representation of OxLDL influx and inhibition of cholesterol efflux in carotid resident macrophages. In the presence of high expression of CD36 in plaque lesion areas, the observed strong correlation between CD36, SRA, and ADFP suggests that carotid lesion resident macrophages are subjected on the one hand to greatly enhanced OxLDL uptake by CD36 and SRA and on the other to inhibition of cholesterol efflux by ADFP. The cholesterol efflux from resident macrophages is probably even further reduced as indicated by the inverse correlation of CD36 expression with plasma cholesterol acceptor ApoAI.

component (IL-8) that is correlated to CD36 in PLA is also observed. This brings a further dimension to the capacity of resident macrophages to attract further monocytes to the sites of inflammation through the release of IL-8. An additional interesting finding in this study is that the plasma acceptor ApoAI, a constituent of HDL, needed for transportation of cholesterol from macrophages is significantly inversely correlated to CD36 gene expression level in PLA. Macrophages do not synthesise ApoAI and need to have it readily available in plasma as a cholesterol acceptor. In the absence of ApoAI, cholesterol efflux is greatly reduced. HDL levels were also inversely correlated to CD36 gene expression but this correlation did not reach statistical significance.

CD36 in this study is statistically more highly expressed in PLA (grade V and VI lesions, AHA classifications) [16] compared to NPLA regions. Cells expressing CD36 are mostly macrophages as indicated by CD68 labelling. CD36 in murine ApoE<sup>-/-</sup> macrophages, compared to ApoE<sup>-/-</sup> CD36<sup>-/-</sup>, has been shown in several studies to be linked to the generation of atherosclerotic lesions. However, a certain caution needs to be taken in trying to understand human atherosclerotic lesions compared to those generated in ApoE<sup>-/-</sup> mice. Indeed, in the ApoE<sup>-/-</sup> murine model of atherosclerosis there is an absence of myocardial infarction or stroke that are the results of clinical complications of atherosclerosis in patients. The intense CD36 labelling obtained in this study for PLA compared to NPLA suggests that this scavenger receptor is actively involved in the perpetuation of lesions in human vessels.

The PMA-differentiated THP-1 incubated with OxLDL showed a similar upregulation of CD36, ADFP, and IL-8 gene and protein expressions compared to those obtained in PLA versus NPLA. In contrast, SRA which is enhanced in PLA versus NPLA was shown to be decreased by OxLDL

treatment in PMA-differentiated THP-1. It is conceivable that SRA and CD36 might be regulated differentially and therefore could play different roles in the formation of foam cells in atherosclerotic lesions. Indeed, CD36 and SRA are differently expressed in lesion area present in post-mortem descending human thoracic aorta [20]. Alternatively, a monocytic cell line such as THP-1 cells may differ in its response from normal monocytes/macrophages.

A certain caution needs to be taken in interpreting the above data. However, an interesting working hypothesis would rely on carotid resident macrophages to take up OxLDL via upregulated CD36 and SRA and inhibit cholesterol efflux as a result of the high expression of ADFP and low levels of plasma cholesterol acceptor ApoAI. Moreover, IL-8 would be implicated in monocyte recruitment to the sites of inflammation (Figure 5).

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