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Role of the Scavenger Receptor CD36 in Accelerated Diabetic Atherosclerosis

Miquel Navas-Madroñal [1,†], Esmeralda Castelblanco [3,4,†], Mercedes Camacho [1,2], Marta Consega l[1], Anna Ramirez-Morros [5], Maria Rosa Sarrias [6], Paulina Perez [7], Nuria Alonso [4,5], María Galán [1,2,*] and Dídac Mauricio [3,4,*]

1 Sant Pau Biomedical Research Institute (IIB Sant Pau), Hospital de la Santa Creu i Sant Pau, Barcelona, Spain
2 Center for Biomedical Research on Cardiovascular Disease (CIBERCV), Madrid, Spai
3 Department of Endocrinology & Nutrition, Hospital de la Santa Creu i Sant Pau & Sant Pau Biomedical Research Institute (IIB Sant Pau), Barcelona, Spain
4 Center for Biomedical Research on Diabetes and Associated Metabolic Diseases (CIBERDEM), Barcelona, Spain
5 Department of Endocrinology & Nutrition, University Hospital and Health Sciences Research Institute Germans Trias i Pujol, Badalona, Spain
6 Innate Immunity Group, Health Sciences Research Institute Germans Trias i Pujol. Center for Biomedical Research on Liver and Digestive Diseases (CIBEREHD), Madrid, Spain.
7 Department of Angiology & Vascular Surgery, University Hospital and Health Sciences.Germans Trias i Pujol, Autonomous University of Barcelona, Badalona, Spain
† These authors contributed equally to this study.
* Correspondence: didacmauricio@gmail.com (D.M.); mgalana@santpau.cat (M.G.); Tel.: +34 93 556 56 61 (D.M.); Tel: +34 93 556 56 22 / 5711 (M.G.); Fax: 93 556 56 02 (D.M.); Fax: +34 93 556 55 59 (M.G.)

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Abstract: Diabetes mellitus entails increased atherosclerotic burden and medial arterial calcification but the precise mechanisms are not fully elucidated. Our aim was to investigate the implication of CD36 in inflammation and calcification processes orchestrated by vascular smooth muscle cells (VSMCs) under hyperglycemic and atherogenic conditions. We examined the expression of CD36, pro-inflammatory cytokines, endoplasmic reticulum (ER) stress markers and mineralization-regulating enzymes by RT-PCR in human VSMCs, cultured in medium containing normal (5 mM) or high glucose (22 mM) for 72 h with or without oxLDL (24 h). The uptake of DiI-labelled oxLDL was quantified by flow citometry and fluorimetry and calcification assays were performed in VSMC cultured in osteogenic medium and stained by alizarin red. We observed an induction in the expression of CD36, cytokines, calcification markers and ER stress markers under high glucose that was exacerbated by oxLDL. These results were confirmed in carotid plaques from subjects with diabetes versus non-diabetic subjects. Accordingly, the uptake of DiI-labelled oxLDL was increased after exposure to high glucose. Silencing of CD36 abolished the induction of CD36 and reduced the expression of calcification enzymes and mineralization of VSMC. Our results indicate that CD36 signaling is involved in hyperglycemia and oxLDL-induced vascular calcification in diabetes.

Keywords: scavenger receptor CD36; atherosclerosis; diabetes; inflammation; vascular calcification

1. Introduction

Pathologic calcification of cardiovascular structures very frequently occurs in the elderly and in people suffering from atherosclerosis and diabetes and is considered an important marker of cardiovascular morbidity and mortality [1]. Atherosclerosis is a chronic inflammatory disease involving endothelial dysfunction, recruitment of inflammatory cells, local production of cytokines, and lipid accumulation within the intima of the vessel wall [2]. Inflammation and dyslipidaemia play crucial synergistic roles in the deterioration of the vascular wall that occurs during the progression of atherosclerosis [3]. Although most studies on atherosclerosis are focused on the role of

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macrophages, vascular smooth muscle cells (VSMC) also play a decisive and, probably, understudied role. VSMC-derived foam cells in human atherosclerotic lesions are well documented [4,5], with recent studies showing that as many as 50% of foam cells in human and murine lesions are derived from VSMC [6,7]. VSMC possess remarkable plasticity with the ability to reprogram their expression pattern as contractile, synthetic, osteochondrogenic, and macrophage-like phenotypes. This is particularly relevant in human arteries which are enriched in VSMC. Migration, proliferation, and synthesis of extracellular matrix by VSMC contribute to early development of lesions [8]. The secretion of proliferative and inflammatory cytokines by VSMC promulgate autocrine activation of VSMC and recruitment of macrophages into the lesion in a paracrine manner [9]. Scavenger receptors mediate the uptake of oxidized low density lipoprotein (oxLDL) by macrophages, resulting in foam cell formation that represents the hallmark of early atherosclerosis [10,11]. Regulation of lipid uptake in VSMC is poorly characterized and less understood than in macrophages, despite being a crucial event in atherogenesis [12]. Vascular smooth muscle cells express scavenger receptors for oxLDL uptake [13,14], and acquire macrophage-like phenotypes upon lipid loading by expressing macrophage markers and suppressing VSMC markers [15,16]. Because they cannot egress from the plaque, uptake of excess lipid by medial and intimal VSMC leads to plaque progression, apoptosis, and eventual plaque instability [17].

Recent work has suggested that there are significant differences in gene expression and modes of lipid loading between VSMC and macrophages [18-20]. Studies in vitro have shown lipid loading by VSMC of cholesterol and oxLDL through scavenger receptor CD36 [16,17], but

mechanisms by which VSMC take up lipids in vivo are mostly unknown.

Vascular calcification (VC) is a common feature in people with type 2 diabetes mellitus and has been shown to be an independent marker for mortality in subjects with advanced cardiovascular diseases [21,22]. Under hyperglycemic conditions, VSMC transit markedly into osteoblast-like cells and increased calcific nodules in vitro [23]. VC induced by high glucose (HG) is considered an active pathological process that involves the crosstalk between cells and the extracellular matrix of the arteries that resembles physiological bone formation [21,24]. The process has many features comparable to embryonic bone formation and involves VSMC transdifferentiation into osteoblast-like cells [24,25]. High glucose not only induces VSMC calcification, but also results in triggering inflammation and endoplasmic reticulum (ER) stress which contribute, at least in part, to the development of VSMC calcification [23,26].

In this study, we aimed to investigate the contribution of CD36 to VSMC induced calcification under hyperglycemic and atherogenic conditions and the mechanisms involved in this process.

2. Results

2.1. Hyperglycemic and Atherogenic Conditions Synergistically Induced the Expression of Scavenger Receptor CD36, and Exposure to High Glucose Enhances oxLDL Uptake in VSMC

To determine whether high glucose concentration induce CD36 expression, we incubated the VSMC with 5 mM, 15 mM, 22 mM and 30 mM of glucose for 72 h. CD36 mRNA levels were gradually increased in response to the increasing concentrations of glucose (Fig S1). For the current study, we selected 22 mM as our HG condition since this is a concentration within ranges of poorly controlled subjects with diabetes. To determine whether exposure to HG in combination with oxLDL could induce a synergistic effect on CD36 expression, primary human VSMC were incubated with medium supplemented with 5 mM or with 22 mM for 72 h and with oxLDL for the last 24 h. We observed that the increase of CD36 mRNA levels in response to HG was significantly enhanced by the addition of oxLDL (Fig. 1A). We next studied the induction of inflammatory markers in VSMC under these hyperglycemic and atherogenic conditions. In addition to CD36, MCP-1, IL-6 and IL-1β mRNA levels were also triggered with HG and in the case of MCP-1, the co-incubation with oxLDL potentiated the induction (Fig. 1B-D).

We then investigated whether the CD36 ligand scavenger protein AIM or CD5L, which is traditionally expressed by macrophages, could play a role in the modulation of CD36 expression and

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its responses to oxLDL, as it does in macrophages [33]. We found that CD5L gene expression was undetectable in VSMC cells (data not shown). We analyzed the induction of CD36 and inflammation markers expression by HG and oxLDL in VSMC co-incubated with the recombinant protein CD5L (rCD5L) or with same concentration of albumin as a control, and we observed that rCD5L drastically reduced the CD36 gene expression in VSMC incubated with HG with or without oxLDL but it did not affect to the expression of MCP-1, IL6 or IL-1β (Fig. S2).

ER stress, as a pathological process involved in diabetes and atherosclerosis, is induced by HG in VSMC [24]. Our results revealed an induction of ER stress markers expression (ATF6, DDIT3, HSPA5 and ERN1) in VSMC stimulated with HG that was exacerbated by the co-incubation with ox-LDL (Fig. 1E-H).

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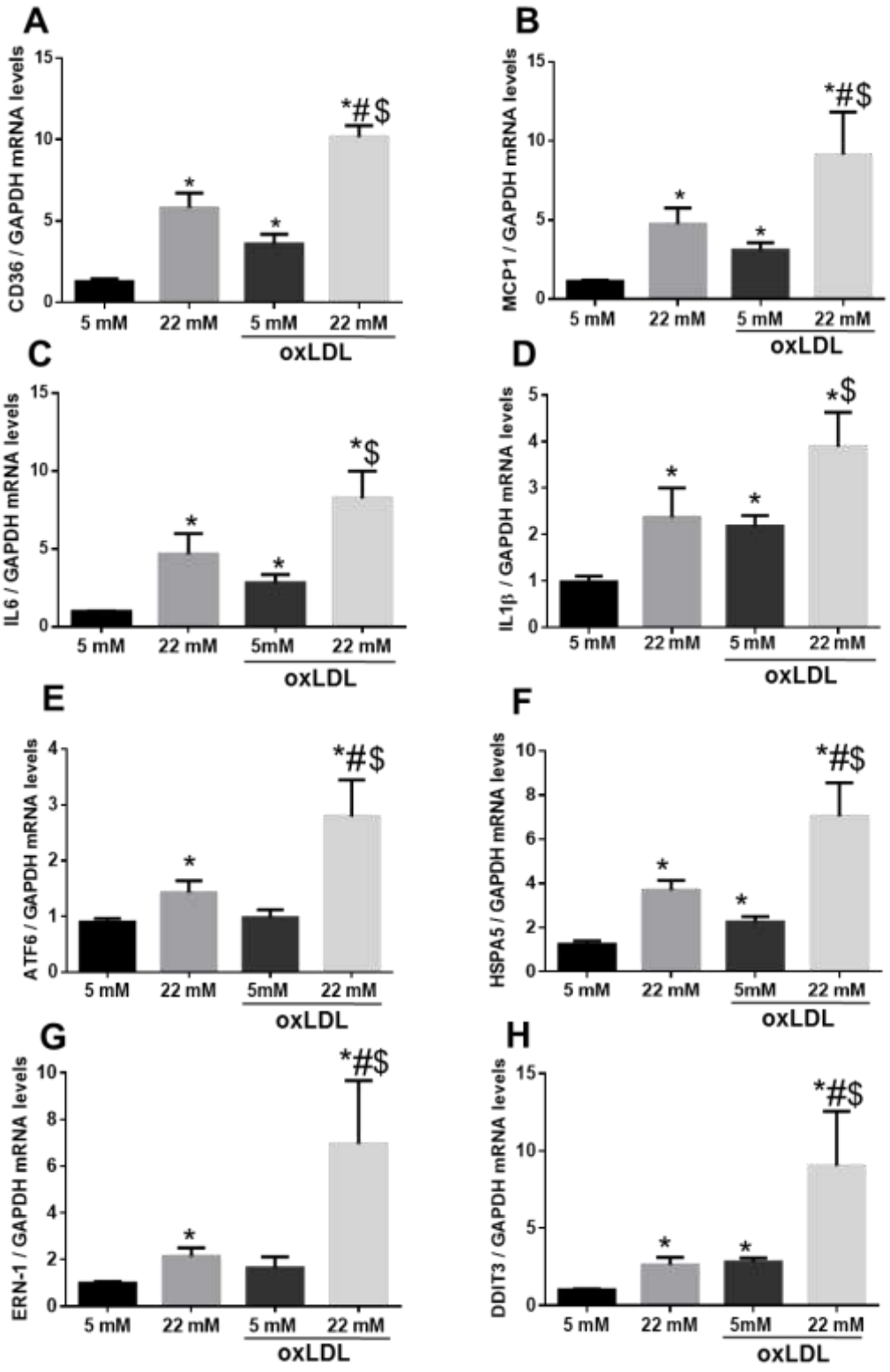

Figure 1. High Glucose and oxLDL synergize in increasing CD36 expression in VSMC and trigger oxLDL uptake. Starved VSMC were cultured for 72 h with M199 medium containing 5 mM (normal glucose, NG) or 22 mM of glucose (high Glucose, HG) and treated or not with 50 μg/ml oxLDL for the last 24 h. **A-H)** CD36, MCP1, IL6, IL-1β and ER stress markers (ATF6, ERN-1, HSPA5 and DDIT3) mRNA levels were determined by quantitative real Time PCR analysis (qPCR) and normalized to GAPDH. VSMC from at least four different donors were used. Mean fold change relative to untreated VSMC-NG ± SEM from five independent experiments performed in duplicate are shown. *$p \leq 0.05$ vs 5 mM; #$p \leq 0.05$ vs 22 mM; \$p≤ 0.05 vs 5 mM+oxLDL.

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To determine whether the scavenger receptor CD36 increased expression is paralleled to an increase of oxLDL uptake in human VSMC under hyperglycemic condition, cells stimulated with HG for 72 h were cultured with fluorescently labeled oxLDL (DiI-oxLDL) for the last 24 h. Lipid uptake, quantified by flow citometry (Fig. 2A) and by fluorimetry (Fig. 2B), showed that exposure to HG significantly increased oxLDL uptake by 48 % (HG DiI-oxLDL vs NG DiI-oxLDL, Fig. 2A) and by 43 % (HG DiI-oxLDL vs NG DiI-oxLDL, Fig. 2B), respectively.

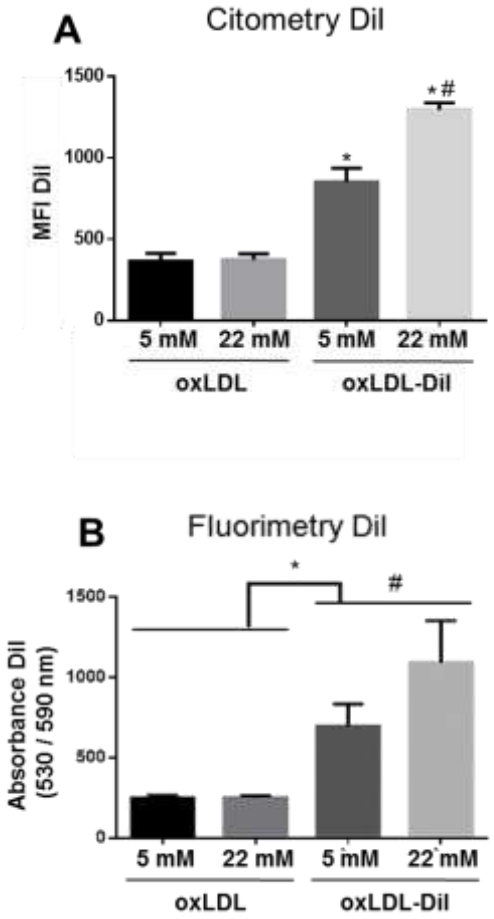

Figure 2. High Glucose triggers oxLDL uptake by VSMC. OxLDL uptake was analyzed by flow cytometry **(A)** and by fluorimetry **(B)** in VSMC incubated for 72 h with NG or HG (22 mM) and treated with dil-oxLDL (5 µg / mL) for the last 24 h. Cells incubated with unlabelled oxLDL were taken as control in flow cytometry and in fluorimetry assays. Mean fold change relative to VSMC-NG ± SEM from four independent experiments performed in duplicate are shown. *$p \leq 0.05$ vs non-labelled oxLDL; #$p \leq 0.05$ vs 22 mM. MFI: Median Fluorescence Intensity.

2.2. Exposure to HG Triggers Calcification in VSMC

OxLDL has been proven to play an essential role in arterial calcification development but the mechanisms by which hyperglycemia accelerates this process remain to be clarified. We studied the effect of the exposure to HG (22 mM) with or without oxLDL in the expression of pro-calcification markes such as alkaline phosphatase (ALPL), secreted phosphoprotein 1 (SPP1, also known as osteopontin) and bone morphogenetic protein 2 (BMP2) in VSMC. After 3 days (Fig. S3) and 7 days (Fig. 3A-C) of exposure to HG, we observed that the expression of ALPL, SPP1 and BMP2 was significantly increased by HG, and it was further potentiated in combination with oxLDL. High glucose-induced-CD36 expression was sustained after 7 days (Fig. 3D). This was accompanied with an increase in calcium depots formation in VSMC cultured in osteogenic medium for 7 days and exposed to HG in comparison with NG condition with or without oxLDL co-incubation (Fig. 3E).

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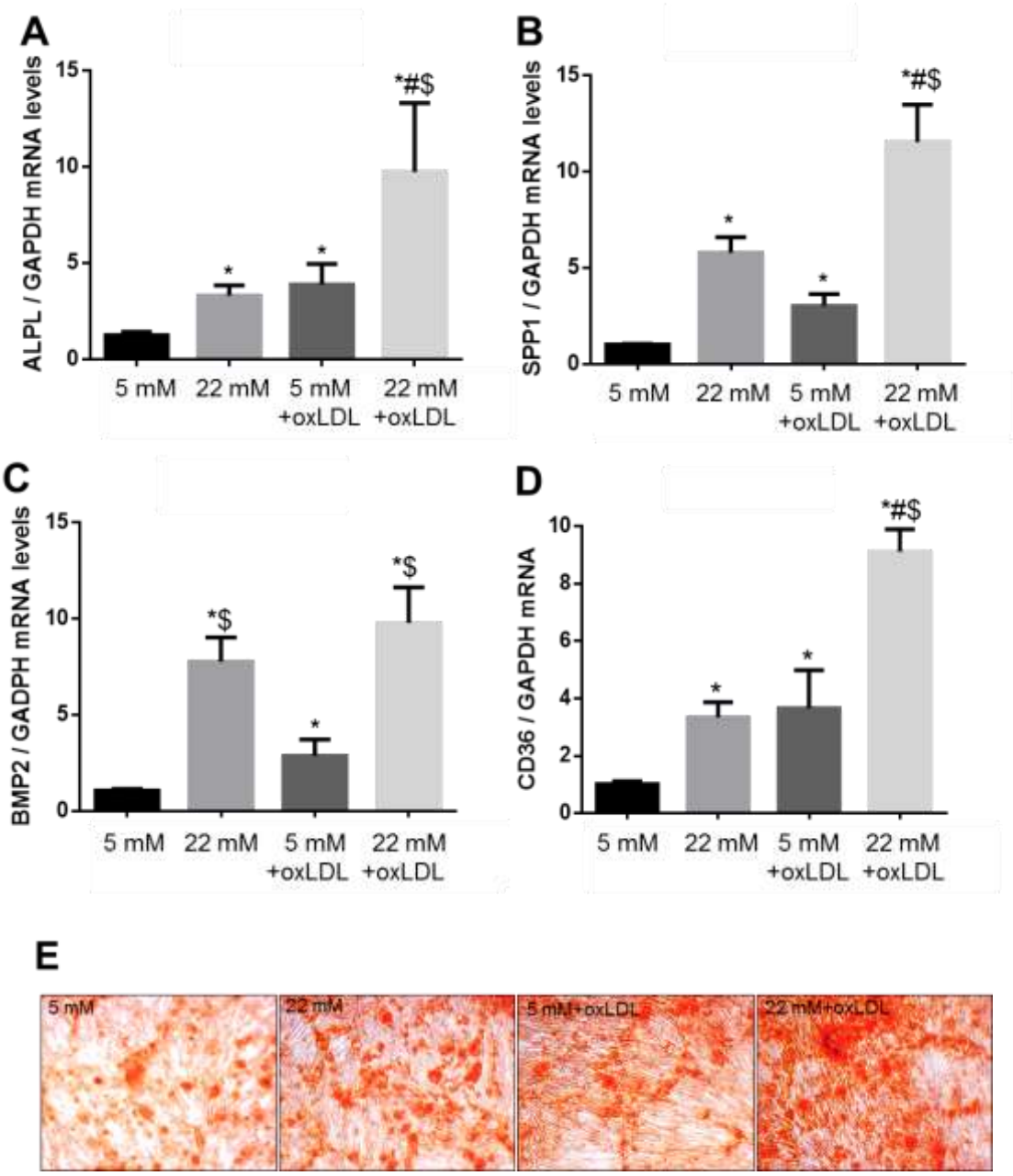

Figure 3. Calcification markers are induced in long-term exposed-VSMC to HG in the absence or presence of oxLDL. The expression of bone matrix proteins that regulate the calcification process were induced in VSMC cultured with high glucose for 7 days and co-incubated or not with oxLDL for the last 24 h. **A-D)** Alkaline phosphatase (ALPL), Bone morphogenetic protein 2 (BMP2), Osteopontin (SPP1) and CD36 mRNA levels were determined by qPCR analysis and normalized to GAPDH. **E)** Representative pictures of VSMC cultured in osteogenic medium for 7 days with NG or HG ± oxLDL and stained with alizarin red. Mean fold change relative to cultured VSMC in NG medium ± SEM (N=5). *$p \leq 0.05$ vs 5 mM; #$p \leq 0.05$ vs 22 mM; \$$p \leq 0.05$ vs 5 mM+oxLDL.

2.3. Silencing of CD36 Decreased ox-LDL Uptake and Limited Osteoblastic Differentiation of VSMC

We tested the mediation of CD36 on oxLDL uptake in VSMC by silencing CD36 gene expression that was assessed by mRNA levels quantification and protein expression. As depicted in figures 4A and 4B, siRNA targeting CD36 completely abolished mRNA expression, reduced protein levels in VSMC and prevented the increase of DiI-oxLDL uptake under HG condition by flow cytometry (Fig. 4C). However, it did not alter the induction of pro-inflammatory markers expression in the absence or in the presence of oxLDL in comparison with VSMC transfected with siRNA CT (Fig. 4D-F).

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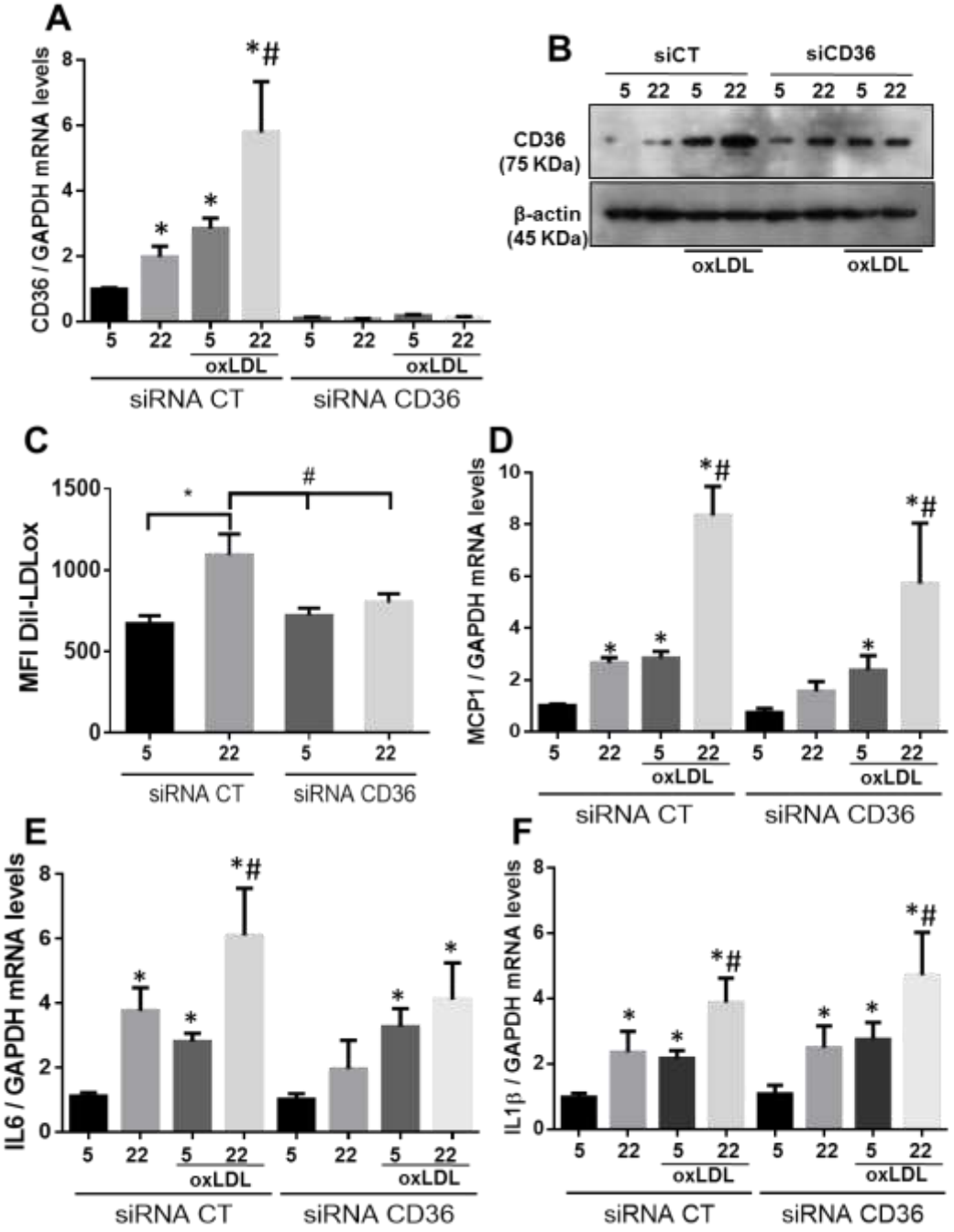

Figure 4. Silencing of CD36 gene expression blunts oxLDL uptake by VSMC but does not affect inflammatory markers expression. A-B) CD36 mRNA levels quantification (N=5) and representative blot of CD36 protein (N=3) in VSMC transfected with siRNA-CD36 or siRNA-CT. C) Quantification of dil-oxLDL uptake by flow cytometry in VSMC transfected with siRNA-CT or siRNA-CD36 (N=4). D-F) MCP-1, IL6 and IL-1β mRNA levels were determined by qPCR analysis and normalized to GAPDH in VSMC transfected with siRNA-CT or siRNA-CD36. Mean fold change relative to VSMC transfected with siRNA-CT cultured under NG condition ± SEM (N = 5). *$p \leq 0.05$ vs 5 mM siRNA-CT or siRNA-CD36; #$p \leq 0.05$ vs 22 mM siRNA-CT or siRNA-CD36.

We further studied whether silencing of CD36 affected pro-calcification markers expression and found that this was significantly reduced in human VSMC exposed to HG for 7 days with or without oxLDL in comparison with cells transfected with control siRNAs (Fig. 5A-C). We then determined whether CD36 is involved in HG and oxLDL-induced osteoblastic differentiation of human VSMC exposed to NG or HG for 7 days in an osteogenic medium. As shown in figures 4D-F, HG and oxLDL

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treatment significantly induced calcium depot formation assessed by alizarin red staining, and this was attenuated by CD36 silencing. However, the effects of transient silencing of CD36 were mostly lost after 10 days of culturing cells in osteogenic medium (Fig. S4).

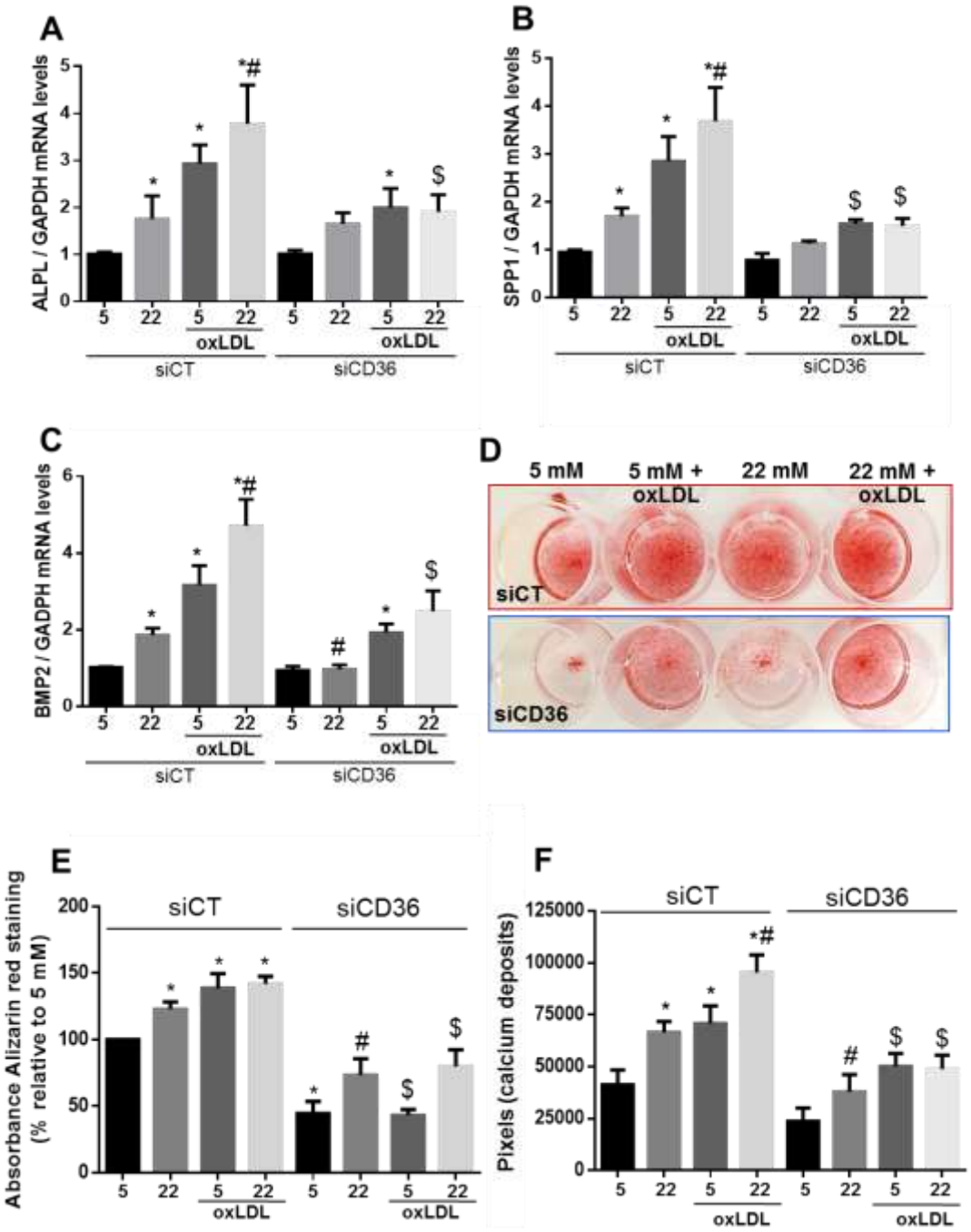

Figure 5. Silencing of CD36 reduces the effect of HG and oxLDL on VSMC calcification. **A-C)** ALPL, BMP2 and SPP1 mRNA levels quantified by qPCR analysis in VSMC cultured with NG or HG condition and treated or not with oxLDL after silencing CD36 (N=5). **D)** Representative picture of stained with alizarin red and fixed VSMC that were transfected with siRNA-CT or siRNA-CD36 and cultured in osteogenic medium after 7 days with NG or HG ± oxLDL.**E)** Quantification of alizarin red staining by colorimetry (N=5). **F)** Quantification of alizarin red staining by imaging analysis (N=5). Results are expressed as mean ± SEM. *p<0.05 *vs*. 5 mM siRNA-CT or siRNA-CD36; #p<0.05 *vs*. 22 mM siRNA-CT; \$$p$<0.05 *vs*. 5 mM+oxLDL siRNA-CT or 22 mM+oxLDL siRNA-CT.

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2.4. Expression of CD36 and ER Stress Indicators in Carotid Plaques from Subjects with and without Diabetes

We aimed to determine whether CD36 expression was altered in carotid plaques from subjects with diabetes (n=15) when compared with plaques from subjects without diabetes (N=15). Demographic and clinical data from participants included in this study are shown in Table 1. We measured gene and protein expression of CD36 by real Time PCR, immunohistochemistry and western blot respectively, and observed that it was markedly increased in plaque tissue from subjects with diabetes (Fig. 6A-C). We aimed to measure circulating soluble CD36 levels in plasma from the subjects included in this study but we could not detect it in most of the samples (data not shown).

Serum oxidized sterols (oxysterols) are a major component in oxidized LDL and are thought to mediate its lipotoxic effects. Oxysterols such as 7-ketocholesterol (7-KC) are abundant in advanced atherosclerotic lesions [34]. Therefore, we measured the levels of free 7-KC levels in the plasma of subjects and observed that circulating 7-KC levels were significantly higher in subjects with diabetes compared to subjects without diabetes (Fig. 6D).

In addition, the gene expression of ER stress markers that represent the three pathways of the unfolding protein response (UPR) was analyzed in these samples. We observed that IRE1, ATF6, CHOP, HSPA5 and ATF4 mRNA levels were significantly increased in tissues from subjects with diabetes, and this was supported by an increased expression of ATF6 and CHOP protein expression when compared with tissue from subjects without diabetes (Fig. 6E-J).

Table 1. Clinical characteristics of the study groups.

	No Diabetes N=18	**Diabetes N=18**	**p.overall**
Sex, men	16 (88.9%)	16 (88.9%)	1.000
Age, years	73.5 [68.0;79.5]	76.0 [71.0;79.8]	0.427
Hypertension	10 (55.6%)	16 (88.9%)	0.063
Dyslipidaemia	16 (88.9%)	18 (100%)	0.486
BMI, Kg/m^2	25.8 (3.47)	27.4 (3.76)	0.188
Smoking:			0.582
No	3 (17.6%)	5 (27.8%)	
Yes	4 (23.5%)	2 (11.1%)	
Former smoker	10 (58.8%)	11 (61.1%)	
sBP, mmHg	140 [130;148]	130 [113;145]	0.145
dBP, mmHg	78.7 (8.31)	70.5 (11.7)	0.022
Antiplatelet treatment	14 (77.8%)	17 (94.4%)	0.338
Statin treatment	16 (88.9%)	16 (94.1%)	1.000
Glucose, mg/dL	94.5 [85.0;111]	133 [119;160]	0.001
Creatinine, mg/dL	0.94 (0.39)	0.98 (0.37)	0.784
eGFR,mL/min/173m^2	82.8 [60.5;90.0]	79.0 [57.0;90.0]	0.647
ALT, U/L	18.0 [16.0;30.8]	20.5 [15.5;26.8]	0.727
GGT, U/L	35.0 [17.0;52.0]	23.0 [13.0;42.0]	0.208
Triglycerides, mg/dL	86.0 [72.8;105]	102 [81.0;142]	0.227
Total cholesterol, mg/dL	138 [123;147]	112 [102;125]	0.028
HDL cholesterol, mg/dL	43.0 (11.7)	39.7 (9.23)	0.422
LDL cholesterol, mg/dL	80.0 [57.0;93.0]	59.0 [45.0;73.0]	0.099
CRP, mg/L	13.1 [2.20;21.3]	4.76 [1.15;8.75]	0.118
Glycated haemoglobin, %	5.70 [5.50;5.95]	6.70 [6.12;7.50]	<0.001
Glycated haemoglobin, mmol/mol	39.0 [37.0;41.5]	50.0 [43.2;58.0]	<0.001
C peptide, nmol/L	1.02 [0.90;1.64]	1.17 [0.90;1.46]	0.760
Leukocyte count, x10*9/L	8.85 [6.68;9.53]	8.20 [6.27;9.85]	0.743
Hemoglobin, g/dL	13.0 (1.81)	12.2 (1.32)	0.152
Hematocrit, %	39.4 (5.51)	36.5 (3.76)	0.081
Platelets, x10*9/L	195 (52.2)	192 (41.5)	0.882
Albuminuria, mg/L	20.4 [5.45;49.9]	4.40 [2.70;24.4]	0.174
Albumin/creatinine ratio, mg/g	13.9 [5.20;50.5]	11.4 [3.80;24.7]	0.765
Diabetes duration, years	-	12.2 (3.43)	-

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Data are shown as median [interquartile], means (SD) or n (%). BMI, body mass index; sBP, systolic blood pressure; dBP, diastolic blood pressure; eGFR; estimated glomerular filtration rate; ALT, alanine aminotransferase; GGT, Gamma-glutamyl transferase; CRP, C-reactive protein.

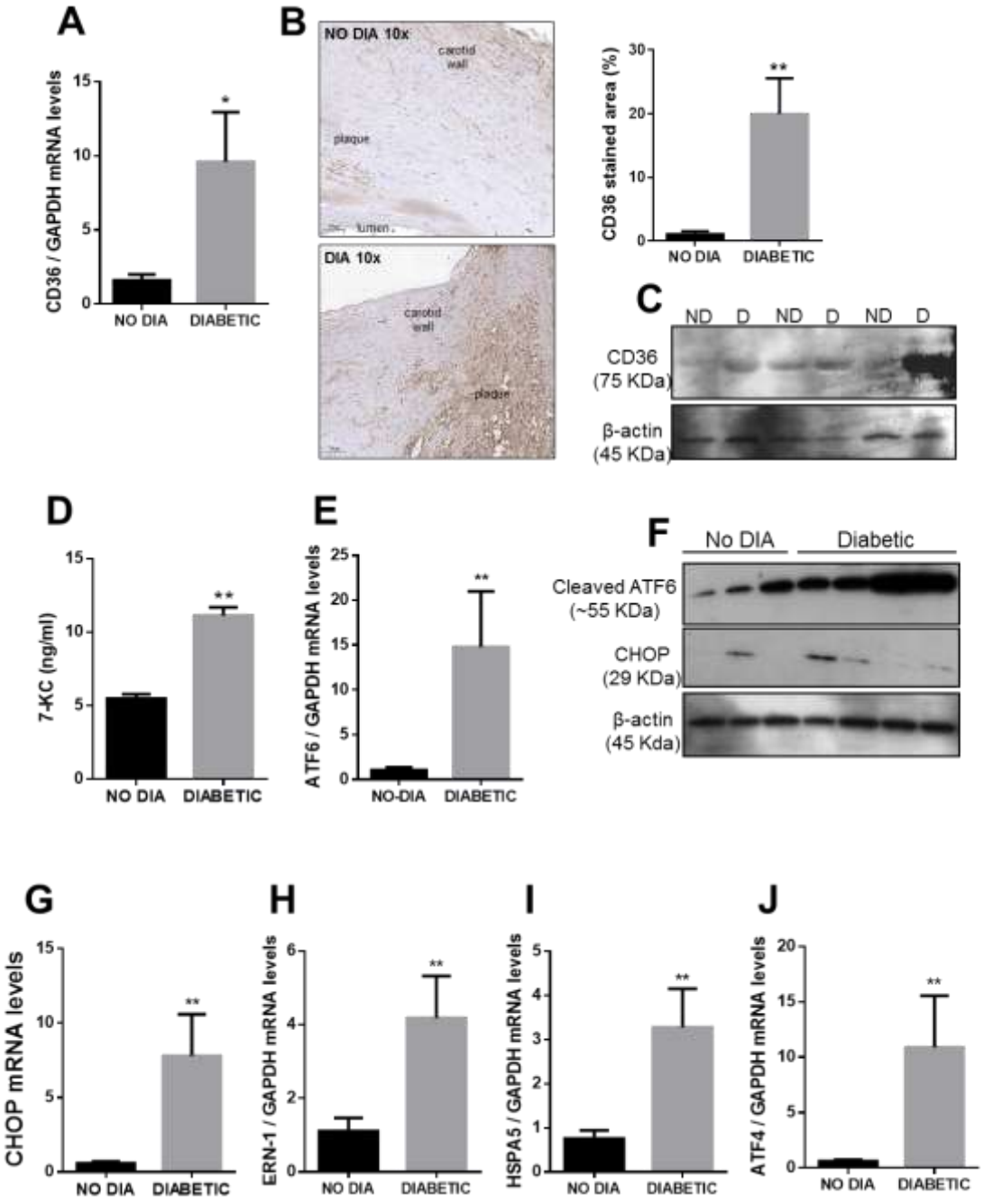

Figure 6. *CD36 and ER stress markers expression in carotid plaques from subjects with and without diabetes.* **A,E,G-J)** the mRNA levels of CD36 and endoplasmic reticulum (ER) stress markers were determined in sections of carotids plaques from subjectswith and without diabetes by qPCR analysis (N=15). **B)** Immunostaining of CD36 inparaffin sections of carotid plaques (N=5). **C)** Representative blot of CD36 in protein lysates from carotid plaques. **F)** Representative blots of ATF6 and CHOP in protein lysates from carotid plaques. **D)** Histogram showing circulating levels of 7-KC in plasma fromsubjects with diabetes (N=18) and subjects without diabetes (N=18) determined by LC-MS/MS. Results are expressed as mean ± SEM. *p<0.05 *vs*. Subjects without diabetes (NO DIA). **p<0.01 *vs*. NO DIA.

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3. Discussion

In the present study, we show that hyperglycemia increased the expression and activity of the oxLDL scavenger receptor-CD36, induce inflammatory and ER stress markers expression and we demonstrate the contribution of CD36 to *in vitro* calcification in human VSMC. These effects were synergistically enhanced by oxLDL. In addition, we found that CD36 expression was increased in carotid plaques from subjects with diabetes compared with carotid plaques from normoglycemic subjects.

Understanding the mechanisms of VC in the progress of diabetes is of high clinical relevance, above all regarding people with diabetes suffering from atherosclerosis. Inflammation and dyslipidemia play crucial synergistic roles in the deterioration that occurs during the progression of atherosclerosis [2,3]. Type 2 diabetes is characterized by elevated serum inflammatory markers and intimal vascular calcification. In agreement with this, we found an exacerbation of the expression of CD36 together with the increase in the expression of pro-inflammatory markers IL-1β, IL6 and MCP-1 in VSMC exposed to HG in the absence or presence of oxLDL. Our results agree with Lopez-Carmona et al that reported an increase of CD36 mRNA expression in peripheral blood mononuclear cells (PBMC) exposed to high glucose and observed a progressive CD36 overexpression in PBMC isolated from pre-diabetic and diabetic individuals with atherosclerosis [35].

The uptake of Di-oxLDL by VSMC elicited by HG, was blunted after silencing CD36 gene expression indicating that the scavenger receptor CD36 was responsible, at least in part for the oxLDL uptake. However, the expression of inflammatory markers triggered by HG were not reduced by CD36 silencing suggesting that the up-regulation of lipid uptake system / machinery is independent of the pro-inflammatory pathways activation in VSMC. In contrast to our results, other authors stated that LDL uptake by VSMC does not occur through mechanisms involving scavenger receptors [36,37]. We believe that this discrepancy may be explained by the fact that VSMC cultured under normal conditions have low abundance of receptors for oxLDL, but according to our data, a chronic exposure to HG triggered CD36 receptor expression which was further exacerbated in response to oxLDL. Under inflammatory conditions, tissue macrophages secrete the protein CD5L which increases macrophage foam cell formation and CD36-mediated oxLDL uptake [33]. CD5L has been implicated in the pathogenesis of several infectious, atherosclerosis [38], and linked to insulin resistance in obesity [39]. We did not detect CD5L expression in VSMC; however, the co-incubation of VMSC with human recombinant CD5L protein prevented the induction of CD36 expression whereas it did not modify the expression of inflammatory markers, thus suggesting that CD5L may play a potential protective role by preventing oxLDL uptake through CD36 by VSMC.

Besides inflammation, chronic induction of ER stress plays a key role in the development of atherosclerosis in vascular tissues and in type 2 diabetes in experimental models [23,40,41]. Unfolding protein response (UPR) signaling in response to ER stress contributes to vascular cell death and to VC [42,43]. In particular, CHOP-mediated cell death is widely characterized in atherosclerosis and is a crucial contributor to the development of advanced atherosclerotic lesions [43,44]. Accordingly, we found that CHOP and other ER stress markers were up-regulated in VSMC after chronic exposure to HG and to oxLDL and in atherosclerotic plaques from subjects with diabetes compared to those without diabetes. In line with our results, other authors previously reported that the UPR is activated in cholesterol-loaded macrophages and that ER stress inhibition effectively reduced foam cell formation and apoptosis through the decrease in the expression of CD36 and oxLDL uptake [45, 46].

The mechanisms underlying the molecular pathogenesis of VC in diabetes are extremely complex and are influenced by the interaction among several inflammatory factors, lipids and oxidative stress. The expression of OPN, BMP2 and ALPL was induced after chronic exposure to HG with or without oxLDL, indicating that the transdifferentiation process into osteoblast-like cells was induced in VSMC. In agreement with previous studies [22-24], we observed that calcification of VSMC cultured under hyperglycemic condition was higher than in those cultured under NG condition in the presence or absence of oxLDL and after 7 or 10 days growing in osteogenic medium. Interestingly, silencing of CD36 not only reduced significantly the expression of calcification markers but also limited the induced-calcification on VSMC as showed by alizarin red staining. Taken

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together, these data suggest that CD36 mediates hyperglycemia and oxLDL-induced osteoblastic differentiation of VSMC. Supporting our data, Staines et al showed that CD36 was among novel differentially expressed genes during the process of bone mineralization in primary osteoblasts and its up-regulation was coupled with a significant increase in mRNA expression of key genes associated with osteoblast mineralization [47].

Oxysterols induce not only apoptosis of vascular cells and macrophages but also induce calcification and osteoblastic differentiation of VSMC [48,49]. Recent publications emphasize the importance of circulating oxysterols in the development of cardiovascular diseases, and in particular the augmentation of 7-KC levels has been associated with cardiovascular events [50]. Our results regarding the circulating 7-KC levels in the subject cohort suggest that the diabetic condition is worsening the oxysterols profile in blood and this may also contribute to accelerate the calcification process of arteries.

In conclusion, our findings point out an important role of CD36 in mediating the deleterious effect of hyperglycaemia and atherogenic lipid molecules on the inflammatory and calcification processes of VSMC involved in the accelerated atherogenesis accompanying these metabolic conditions. Most importantly, the present study suggests that CD36 and ER stress could be potential targets for novel therapeutic strategies to alleviate atherosclerosis in subjects with diabetes.

4. Methods

4.1. Human Samples

In this single-center, observational study, plasma samples and carotid plaques from symptomatic (cerebrovascular disease event) and asymptomatic subjects undergoing carotid surgical endarterectomy for carotid disease performed at University Hospital Germans Trias i Pujol (Badalona, Spain) were collected between March 2014 and March 2018. This research was approved by the institutional ethics committee of Hospital Germans Trias i Pujol and conducted in accordance with the Declaration of Helsinki; all participants signed informed consent forms. Demographics, risk factors, and clinical characteristics were recorded for all subjects. In this study, we included 36 subjects who were stratified into two groups: subjects with diabetes (n=18) and subjects without diabetes (n=18). Only 15 samples of tissue embedded in OCT were available per group.

All carotid endarterectomies were performed to relieve significant carotid stenosis, as established by the North American Symptomatic Carotid Endarterectomy Trial (NASCET), as previously published [27,28]: 50–99% luminal narrowing in people with symptomatic carotid stenosis and ≥ 70% in those with asymptomatic carotid stenosis.

4.2. Cell Culture

Human VSMC were isolated from abdominal aorta of multi-organ donors by an explant procedure as previously described [29]. Briefly, endothelium denuded medial tissue was cut into 2-4 mm cubes that were transferred to a 25 cm^2 culture flask containing 5 mL of pre-warmed culture medium M199 (Gibco, Carlsbad, CA, USA) supplemented with 10 % foetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 µg/mL streptomycin) (all from Biological Industries, Kibbutz Beit-Haemek, Israel). VSMC migrate out from the explants within 2-3 weeks. The medium was exchanged every 3 days after the onset of cell outgrowth. When a significant outgrowth was reached tissue fragments were collected with forceps and placed in a new dish with fresh medium. Then, after removing the explants from the flask surface, the cells that remain in the dish were cultured until confluence, trypsinised, used as P1 stage cells, and routinely subcultured. Cells used in the present experiments were between the third and sixth passage. VSMC at these passages appeared as a relatively homogeneous cell population, showing a hill-and-valley pattern at confluence. Western blot analysis for specific differentiation markers revealed a clear positive band for α-actin (45 kDa) and calponin (33 kDa). Human VSMC were cultured in medium M199 supplemented with 20% FBS, 2% human serum, 2 mmol/L L-glutamine (Invitrogen), 100 U/mL penicillin G and 100 µg/mL streptomycin. VSMC from 6 different donors were used. The research

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was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Hospital de la Santa Creu i Sant Pau (12/031/1316).

For experimental procedures, cells were seeded in multiwell plates and subconfluent cells were starved in medium supplemented with 1% FBS for 24 h prior to the addition of medium containing 5 mM (NG, normal glucose), 22 mM of glucose (high glucose, HG,) (D-Glucose G7021, Sigma Aldrich) or vehicle (PBS) for 72 h in the presence or absence of 50 μg/mL human purified oxLDL (BT-910 Alfa Aesar J65591) for the last 24 h. Treatments did not induce cytotoxicity analysed by the MTT assay (Roche Diagnostics, Indianapolis, IN, USA).

In additional assays, the cells were co-incubated with 1 μg/mL endotoxin-free recombinant human CD5L (R&D systems, Abingdon, UK) or with 1 μg/mL albumin as control (Sigma-Aldrich) for 48 h.

4.3. Transfections with Small Interfering RNA (siRNA)

We used siRNAs against CD36 and siRNAs control (SMARTpool: ON-TARGET plus CD36 siRNA, L-010206-00-0005 and ON-TARGETplus Non-targeting siRNA #1, D-001810-01-05, Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA) to transfect VSMCs. Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) was used for siRNA delivery. Cells were transfected with 20–50 nM siRNA, using 7.5 μL of Lipofectamine RNAiMAX Reagent following manufacturer's instructions. After transfection (24 h), the medium was replaced and cells were washed twice in culture medium and incubated for at least 8 h in complete fresh medium. Thereafter, VSMC were serum-deprived for 24 h and exposed to NG or HG for 72 h with or without oxLDL for the last 24 h. In calcification experiments and after transfection, VSMC were incubated with osteogenic medium for 7-10 days.

4.4. Total mRNA and Protein Isolation from Tissue and Cells

RNA and protein lysates were obtained from frozen OCT-embedded tissue by previously disolving it in ddH_2O in order to remove the OCT by centrifugation. Total RNA isolation from human VSMC and total RNA and protein isolation from human carotid plaques were performed by using a tissue homogenizer and the Tripure reagent (Roche Diagnostics) following the manufacturer's instructions. RNA integrity was determined by electrophoresis in agarose gels and was quantified by a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, Ma). Protein lysates from cells were prepared in a RIPA buffer (150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 2 mM EDTA, 50 mM Tris-HCl (pH 8) and following a standard protocol.

4.5. Quantitative Real-Time PCR

DNase I-treated total RNA (1 μg) was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) with random hexamers. Quantification of mRNA levels was performed by real-time PCR using pre-designed validated assays (TaqMan Gene Expression Assays; Applied Biosystems) for human CD36 (Hs00169627_m1), ATF6 (Hs00232586_m1), CHOP or DDIT3 (Hs99999172_m1), XBP-1 (Hs00231936_m1), heat shock protein 5 (HSPA5) or GRP78 (Hs99999174_m1), IRE1 or ERN1 (Hs00176385_m1), ATF4 (Hs00909569_g1), CCL-2 or MCP-1 (Hs00234140_m1), IL1β (Hs01555410_m1), IL6 (Hs00174131_m1), ALPL (Hs01029144_m1), BMP2 (Hs00154192_m1), SPP1 (Hs00959010_m1) and CD5L (Hs00935902_m1). As endogenous controls glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02758991_g1) and β-actin (Hs99999903_m1) were used. Each sample was amplified in duplicate. Similar results were obtained after normalisation to either housekeeping gene. Quantitative RT-PCR was carried out in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Relative mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method.

4.6. OxLDL Uptake

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To generate DiI-oxLDL, a stock solution of the fluorescent probe:1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes Invitrogen, Carlsbad, CA) was prepared in DMSO and then added to the LDL solution to yield a final ratio of 1 μg of DiI/mg of oxLDL. The mixture was incubated for 18 h at 37 °C under light protection as previously described [30]. Cells were incubated with HG or NG in M199 medium containing 0.2 % SBF (v/v) for 72 h and with DiI-oxLDL (5 μg/mL) for the last 24 h. To test the specificity of uptake, the VSMC were treated with DiI-oxLDL and an excess of unlabeled oxLDL. After treatments, cells were washed three times with PBS and harvested with trypsin solution (0.25% trypsin, 0.02% EDTA). Then, the cells were centrifuged at 500*xg* for 5 min, washed once with M-199 complete medium at 37 °C and twice with PBS at 500*xg* for 5 min at 4 °C. The uptake of DiI-oxLDL by VSMC was analyzed by flow cytometry on a FACScan flow cytometer (BD Biosciences) with 10,000 events acquired for each sample. Data were calculated and expressed as mean fluorescence intensity (MFI). As negative control, VSMC incubated with un-labelled oxLDL were used.

In parallel, the uptake of DiI labeled oxLDL was quantified by fluorimetry. After VSMC incubation with DiI-labeled oxLDL or with unlabelled oxLDL as stated above, cells were washed 4 times with PBS and solubilized with 250 μL of 1% Triton X-100 in PBS. Culture dishes were shaken for 15 min at room temperature (RT) and supernatant was removed. Fluorescence was measured immediately at 530 nm excitation-light and 590 nm emission-light.

Additional oxLDL uptake experiments were performed after silencing of CD36 expression. Briefly, VSMC were transfected with 20 nM of a set of four siRNAs targeting CD36 or an equal concentration of a non-targeting negative control pool by using Lipofectamine™ RNAiMAX and following the manufacturer's instructions. Twenty-four hours later, the medium was replaced and cells were incubated with HG or NG in M199 medium containing 0.2 % SBF (v/v) for 72 h and with DiI-oxLDL for the last 24 h.

4.7. In vitro Calcification

Calcification of human aortic VSMC was induced by culturing confluent cells in complete medium supplemented with 5 mM β-glycerophosphate (Sigma Aldrich) and 4 mM $CaCl_2$ to achieve maximal mineralization. The calcification medium was changed every 2-3 days. After 7-10 days, cells were fixed in 4 % formaldehyde in PBS for 45 min at 4°C and then they were washed in deionized water and stained with a 2% aqueous solution of Alizarin Red S (Sigma Aldrich) for 5 min. After Alizarin red S staining the wells were washed four times with dH_2O while gentle shaking and the plates were then left at an angle for 2 min to facilitate removal of excess water. Stained monolayers were visualized by phase microscopy using an inverted microscope (Nikon). At least 8 images per well from 5 independent assays were taken and subsequently analyzed to quantify calcium deposits by imaging analysis with Adobe Photoshop.

The recovery and semiquantification of Alizarin red S staining was performed by acetic acid extraction and neutralization with ammonium hydroxide followed by colorimetric detection at 405 nm as previously described [31].

4.8. Western Blot

Tissue lysates were separated by SDS-PAGE and transferred to 0.45 μm polyvinylidene difluoride membranes (Immobilon, Millipore. Merck KGaA, Darmstadt, Germany). Blots were incubated with antibodies directed against CD36 (NB400-144), ATF6 (NBP1-40256) and CHOP (NB600-1335) purchased from Novus Biologicals (Bio-Techne LD-R&D Systems Europe Ltd, Abingdon, UK). Equal loading of protein in each lane was verified by β-actin (A5441, Sigma Aldrich).

4.9. ELISA

The circulating levels of soluble CD36 in plasma from subjects were measured using commercially available ELISA kits (ABE-196-02, Nordic BioSite, Täby, Sweden) in accordance with the manufacturer's instructions.

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4.10. Immunostaining

Human tissue samples of carotid plaques from patients with and without diabetes were fixed in 4% paraformaldehyde/0.1 M PBS (pH 7.4) for 24 h and embedded in paraffin. For immunohistochemistry, tissue sections (5 μm) were deparaffinized in xylene, rehydrated in graded ethanol, and treated with 0.3% hydrogen peroxide for 30 min to block peroxidase activity. Then, samples were blocked with 10% of normal serum and incubated with an antibody against CD36 overnight at 4°C. After washing, samples were incubated for 1 h with a biotinylated secondary antibody (Vector Laboratories, Peterborough, UK). After rinsing 3 times in PBS, standard Vectastain (ABC) avidin-biotin peroxidase complex (Vector Laboratories) was applied, and the slides were incubated for 30 min. Colour was developed using 3,3'-diaminobenzidine (DAB) and sections were counterstained with haematoxylin before dehydration, clearing, and mounting. Negative controls, in which the primary antibody was omitted, were included to test for non-specific binding. Results were quantified and expressed as percentage of positive area *versus* total area in independent sections of carotid plaques.

4.11. Determination of 7-ketocholesterol in Plasma

Plasma was separated from EDTA blood collection tubes within 2 hours of the blood collection. Plasma was frozen in aliquots at -80° C promptly after separation. Absolute quantification of 7-ketocholesterol (7-KC) was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with atmospheric pressure chemical ionization (APCI) interface (UHPLC-(+) APCI-MS/MS) in the Centre for Omic Sciences (Reus, Tarragona, Spain as previously described [32].

4.12. Statistical Analysis

GraphPad Prism 4.0 software (GraphPad, USA) and R statistical software, version 3.3.1 were used for statistical analysis. Data were expressed as mean ± SEM and values of $p \leq 0.05$ were considered significant. When data fitted a normal distribution differences between two groups were assessed using the Student's t test (two-tailed) and one-way ANOVA and the Bonferroni test for more than two groups. When normality failed, we used the Mann-Whitney rank sum test to compare two groups and Kruskal-Wallis one-way analysis of variance on ranks for multiple comparisons (Dunn's method). The descriptive statistics of the mean (standard deviation) or median [interquartile range] of subject characteristics were estimated for quantitative variables with a normal or non-normal distribution, respectively. For the qualitative variables, absolute and relative frequencies were used. The normal distribution was analyzed by the Shapiro-Wilks test. The significance of the differences in qualitative variables was assessed by Chi-squared test or Fisher's exact test.

5. Conclusions

The scavenger receptor CD36 expression and signaling in VSMC is poorly characterized. Our findings reveal a decisive role for the CD36 scavenger in vascular calcification under hyperglycemic condition and indicate potential mechanistic insights into the acceleration of atherosclerosis in diabetic subjects. We demonstrate that chronic exposure of VSMC to HG can accelerate inflammation and calcification in response to atherogenic stimulus through the induction of CD36 scavenger receptor and that ER stress induction may be a mechanism by which CD36 signaling contributes to diabetic atherosclerosis. Altogether, our results indicate that CD36 and ER stress are potential therapeutic targets to alleviate atherosclerosis in diabetes.

Author Contributions: D Mauricio and M Galán conceived, designed and supervised the study. M Navas-Madroñal, E Castelblanco and M Galán performed experiments, analysed and interpreted data. D Mauricio, N Alonso, M Camacho and P Perez were responsible for the clinical aspects of the study, and participated in human material collection and analysis. MR Sarriá, and M Consegal conceived specific experiments, revised and carried out results interpretation. M Galán and D Mauricio drafted the manuscript and were responsible for funding

acquisition. All the authors revised the manuscript for important intellectual content and gave their final approval of the submitted version.

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Abbreviations

ABC: avidin-biotin peroxidase complex

DAB: 3,3′-diaminobenzidine

DiI: 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine perchlorate

ER: endoplasmic reticulum

HG: high glucose

IL: Interleukin

MFI: mean fluorescence intensity

NG: normal glucose

oxLDL: oxidized low density lipoprotein

siRNA: small interference

VC: Vascular calcification

VSMC: Vascular smooth muscle cells.

7-KC: 7-ketocholesterol

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