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# Omega-3 fatty acids attenuate constitutive and insulin-induced CD36 expression through a suppression of PPAR $\alpha/\gamma$ activity in microvascular endothelial cells

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## Summary

Microvascular dysfunction occurs in insulin resistance and/or hyperinsulinaemia. Enhanced uptake of free fatty acids (FFA) and oxidised low-density lipoproteins (oxLDL) may lead to oxidative stress and microvascular dysfunction interacting with CD36, a PPAR $\alpha/\gamma$ -regulated scavenger receptor and long-chain FFA transporter. We investigated CD36 expression and CD36-mediated oxLDL uptake before and after insulin treatment in human dermal microvascular endothelial cells (HMVECs),  $\pm$  different types of fatty acids (FA), including palmitic, oleic, linoleic, arachidonic, eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids. Insulin ( $10^{-8}$  and  $10^{-7}$  M) time-dependently increased DiI-oxLDL uptake and CD36 surface expression (by  $30 \pm 13\%$ ,  $p < 0.05$  vs. untreated control after 24 hours incubation), as assessed by ELISA and flow cytometry, an effect that was potentiated by the PI3-kinase inhibitor wortmannin and reverted by the ERK1/2 inhibitor PD98059 and the PPAR $\alpha/\gamma$  antagonist GW9662. A  $\geq 24$  hour exposure to  $50 \mu\text{M}$  DHA

or EPA, but not other FA, blunted both the constitutive (by  $23 \pm 3\%$  and  $29 \pm 2\%$ , respectively,  $p < 0.05$  for both) and insulin-induced CD36 expressions (by  $45 \pm 27\%$  and  $12 \pm 3\%$ , respectively,  $p < 0.05$  for both), along with insulin-induced uptake of DiI-oxLDL and the downregulation of phosphorylated endothelial nitric oxide synthase (P-eNOS). At gel shift assays, DHA reverted insulin-induced basal and oxLDL-stimulated transactivation of PPRE and DNA binding of PPAR $\alpha/\gamma$  and NF- $\kappa$ B. In conclusion, omega-3 fatty acids blunt the increased CD36 expression and activity promoted by high concentrations of insulin. Such mechanisms may be the basis for the use of omega-3 fatty acids in diabetic microvasculopathy.

## Keywords

Insulin, atherosclerosis, CD36, omega-3 fatty acids, type 2 diabetes, insulin resistance

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## Introduction

Impaired microvascular reactivity with a poor response to vasodilatory stimuli precedes the onset of diabetes and may play a role in its initial development (1–3). Microvascular abnormalities have indeed been found in people at high risk of developing diabetes, such as obese subjects, subjects with impaired glucose tolerance (2, 3) and first-degree relatives of diabetic patients (1, 4).

Among mediators of microvascular dysfunction, CD36 (also called fatty acid transporter, FAT/CD36) has been linked to the uptake of oxidised low-density lipoproteins (oxLDL) (5) and mediates the transmembrane transport of long-chain fatty acids (LCFA) in several cell types, including cardiomyocytes, macrophages, adipocytes and microvascular endothelial cells, whereas it is not expressed in macrovascular endothelial cells (6). Originally identified as a surface glycoprotein on platelets (7), CD36 is

required in macrophages for lipid accumulation in the vessel wall and foam cell formation (8). In monocytes and macrophages the uptake of oxLDL mediated by CD36 is increased by insulin (9). Previous research has shown that insulin-stimulated permanent relocation (increased surface expression) of LCFA transporters, mainly CD36, from intracellular stores to the sarcolemma in cardiomyocytes is accompanied by the accumulation of lipids and lipid metabolites (6, 10), resulting in decreased contractile activity of the heart and diabetic cardiomyopathy (10). On the other hand, it has been shown that total CD36 expression is regulated by FA in other cell types (11). Conditions of insulin resistance/hyperinsulinaemia are characterised by elevated levels of free fatty acids (FFA). In experimental models, an increase of plasma FFA impairs muscle capillary recruitment (12), which may lead to microvascular dysfunction, in turn impairing glucose and insulin delivery to the muscle and insulin sensitivity (12–14). Therefore, CD36 may me-

diate the microvascular damage occurring as a consequence of high levels of FFA. This might occur also because increased endothelial expression of CD36 increases the endocytic uptake of modified forms of low-density lipoprotein (LDL), including oxidised LDL (oxLDL) (5, 15–17), promoting endothelial dysfunction.

We therefore hypothesised that, similarly to what occurs in cardiomyocytes, an increase in CD36 total expression and/or surface presence may occur at the microvascular endothelial level in conditions characterised by compensatory hyperinsulinaemia preceding the onset of diabetes, such as obesity and insulin resistance, playing a role in diabetic endothelial microvascular dysfunction. In this context we also hypothesised that the type of LCFA presented to the cells may affect CD36 expression, and therefore studied the effects of different types of LCFA on the basal and insulin-stimulated expression of CD36 in human microvascular endothelial cells (HMVEC).

## Materials and methods

### Materials

Human recombinant insulin was purchased from Novo-Nordisk Farmaceutici S.p.A. (Rome, Italy). Palmitic acid (PA, 16:0), oleic acid (OA, 18:1 n-9), linoleic acid (LA, 18:2 n-6), arachidonic acid (AA, 20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), were obtained as 99% pure sodium salts from Sigma-Aldrich (St. Louis, MO, USA). The p38 mitogen activated protein kinase (MAPK) inhibitor SB202190 and the phosphoinositol-3 (PI3)-kinase inhibitor wortmannin were also from Sigma. The extracellular kinase (ERK)1/2 inhibitor PD98059 was purchased from Alexis Biochemical (Lausen, Switzerland), the peroxisome proliferator-activated receptor (PPAR)- $\gamma$  antagonist GW9662 from Calbiochem (Gibbstown, NJ, USA) and tumour necrosis factor (TNF)- $\alpha$  from R&D (Minneapolis, MN, USA).

### Cell cultures

Subconfluent human dermal microvascular endothelial cells (HMVEC, purchased from Clonetics, Baltimore, MD, USA) at passage 3 were preincubated in endothelial growth medium (purchased from Clonetics) with FA for 12 hours (h), followed by stimulation with insulin ( $10^{-9}$ – $10^{-7}$  M) for an additional 24 h, after which time cells were collected. In the time-course experiments, cells were incubated with insulin ( $10^{-9}$ – $10^{-7}$  M) for 0–72 h. In the experiments aimed at determining the insulin signalling involved in the insulin effects on CD36 expression, some monolayers were also treated with the PI3-kinase inhibitor wortmannin or the ERK1/2 inhibitor PD98059 or the p38MAPK inhibitor SB202190 or the PPAR $\alpha/\gamma$  antagonist GW9662 for 30 minutes (min), and then incubated with insulin for 24 h. Cell viability after treatments

was assessed by evaluating several parameters, including cell morphology at phase-contrast microscopy, Trypan blue exclusion, and total cellular proteins.

### ELISA

Assays of cell surface CD36 were carried out by an enzyme-linked immunosorbent assay (ELISA), using goat anti-human monoclonal antibody against CD36 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cell monolayers were incubated with saturating concentrations of a specific monoclonal antibody against the target molecule, then incubated with a peroxidase-coupled secondary antibody, washed, and then incubated with the developing solution. Cell monolayers were washed 3x with phosphate-buffered saline (PBS) between each incubation step, and the integrity of the monolayer monitored by phase-contrast microscopy. The reaction was stopped by adding 25  $\mu$ l of 1 M  $H_2SO_4$ , and coloured products were quantified spectrophotometrically at 450 nm. All samples were assayed in 6 replicates, and each experiment was repeated at least 3x. Signals measured were corrected for cell number assessed in a haemocytometer after crystal violet staining (100  $\mu$ l of [0.04% crystal violet in 4%(v/v) ethanol] for 30 min at room temperature). The coloured products were quantified spectrophotometrically at 595 nm; the ratio of absorbance values at 490 and 595 was then measured and used for the analysis.

### Uptake of oxLDL

To determine whether changes in CD36 expression are paralleled by increased oxLDL uptake, cells exposed to insulin and/or FA were treated with 10  $\mu$ g/ml oxLDL conjugated with the red fluorescence probe 3,3'-diiodoacetylindocarbocyanine (DiI-oxLDL, from Kalen Biomedica (Montgomery Village, MD, USA) for 6 h at 37°C in serum-free culture medium. Neutralisation experiments were done by co-incubating cells with insulin and anti-CD36 antibody or control IgG, with the aim of quantitatively evaluating the role of CD36 in the uptake of oxLDL. Visual evaluations were performed by immunofluorescence microscopy, and measurements by microplate fluorimetry (PolarStar Optima, BMG Labtech, San Antonio, TX, USA) and flow cytometry, setting excitation and emission wavelengths at 530 and 590 nm, respectively. Cell number per well was determined by 4',6-diamidino-2-phenylindole (DAPI) staining, setting excitation and emission wavelengths at 350 and 470 nm, respectively.

### Flow cytometry

At the end of incubation with DiI-oxLDL, HMVECs grown in four-well chamber slides were analysed at flow cytometry for determination of surface CD36 expression and DiI-oxLDL uptake. HMVEC were washed with PBS, detached by scraping in 3 mM EDTA-Hanks' balanced salt solution without trypsin, then resus-

pended ( $1 \times 10^6$  cells) in 100  $\mu$ l of PBS/2% FCS-2 mM EDTA and incubated with the goat anti-human monoclonal antibody against CD36 for 60 min at 4°C. After washing, cells were exposed to mouse anti-goat F(ab')<sub>2</sub>, labelled with fluoresceine-isothiocyanate (Immunotech, Cremascoli, Milan, Italy) at 4°C. After further washing and fixation in 4% paraformaldehyde, the cell suspension was passed through a FACScan analyser (Becton-Dickinson Immunocytometry Systems, Mountain View, CA, USA). Results were plotted as intensity of fluorescence (in arbitrary units, on a logarithmic scale, on the abscissa) versus cell number (on the ordinate, total cells counted:  $5 \times 10^3$  for each condition).

### Immunoblotting of total CD36 and phosphorylated eNOS

Total proteins were isolated from HMVEC in ice-cold Radio-Immuno Precipitation Assay (RIPA) buffer. Plasma membrane and cytosolic proteins were separately extracted using a subcellular protein fractionation kit (Pierce, Rockford, IL, USA), according to the manufacturer's protocol. Proteins were separated under reducing conditions and electroblotted onto polyvinylidene fluoride membranes (Immobilon-P+ Millipore, Bedford, MA, USA). After blocking, the membranes were incubated overnight at 4°C with a primary antibody against CD36 (Santa Cruz Biotechnologies) and active phosphorylated eNOS (Cell Signaling, Danvers, MA, USA). The blots were developed using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). Equal protein loading and/or transfer were verified by probing stripped filters with anti- $\beta$ -actin antibody (Sigma Aldrich). The intensity of each immunoreactive protein band was measured by densitometry.

### Assay for nitric oxide (NO) production

The activity of eNOS was determined in the culture medium by assaying nitrites as previously described (18). Nitrites were taken an index of NO production, using the Griess reagent system (Promega, Madison, WI, USA) according to the manufacturer's protocol.

### Electrophoretic mobility (gel) shift assay

Nuclear proteins from stimulated or unstimulated HMVEC were purified as previously described (19). Protein concentration was determined by the Bio-Rad Laboratories Protein Assay (Bio-Rad Laboratories Inc, Hercules, CA, USA). Double-stranded oligonucleotides containing the consensus sequence for PPAR $\alpha/\gamma$  (5'-CAA AAC TAG GTC AAA GGT CA-3'; 3'-GTT TTG ATC CAG TTT CCA GT-5') or the consensus sequences for NF- $\kappa$ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 5'-CCT GGG AAA GTC CCC TCA ACT-3') were end-labelled with [ $\gamma$ -<sup>32</sup>P]-ATP with the T4 polynucleotide kinase (Promega) and purified by Bio-Spin 30 chromatography columns (Bio-Rad). Binding reactions contain-

ing 10  $\mu$ g of crude nuclear extract were performed using the electrophoretic mobility gel shift assay core system (Promega) according to the manufacturer's protocol. The equal loading of nuclear proteins was verified by Western analysis using an antibody against lamin B.

### Immunoblotting of p65 and p50 NF- $\kappa$ B subunits

An aliquot of the nuclear extracts used for EMSA and cytosolic extracts were loaded on 5–10% SDS-polyacrylamide gel electrophoresis under reducing conditions (18) and electroblotted onto polyvinylidene fluoride membranes (Immobilon-P+, Millipore). The membrane was blocked in 3% goat serum for 1 h and incubated overnight at 4°C with the following primary antibodies: 1) polyclonal anti-rabbit p65 and p50 (Santa Cruz); 2) anti- $\beta$ -actin (Sigma); 3) anti-lamin B (Santa Cruz). The blots were developed using a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). The intensity of each immunoreactive protein band was measured by densitometry.

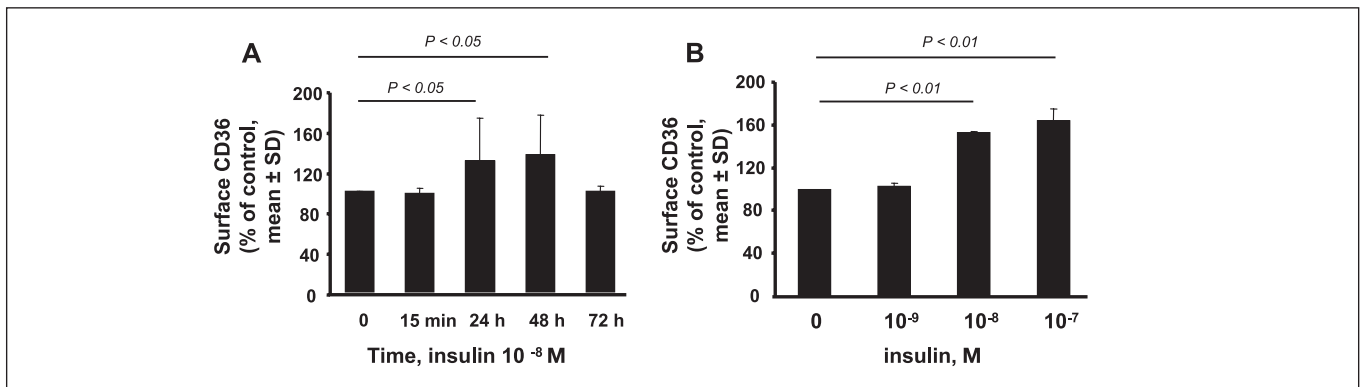
### Statistical analyses

Two-group comparisons were performed by the Student's t-test for unpaired values. Comparisons of means of  $\geq 3$  groups were performed by analysis of variance (ANOVA), and the existence of individual differences, in case of significant F values at ANOVA, tested by Scheffé's multiple contrasts. Comparisons of distribution of fluorescence intensities at flow cytometry were performed by the Kolmogorov-Smirnov statistics with the aid of the 1997 CellQuest<sup>TM</sup> software package from Becton Dickinson. For all tests, statistical significance was set at  $P < 0.05$ . Statistical analyses were carried out with SPSS 15.0 version.

## Results

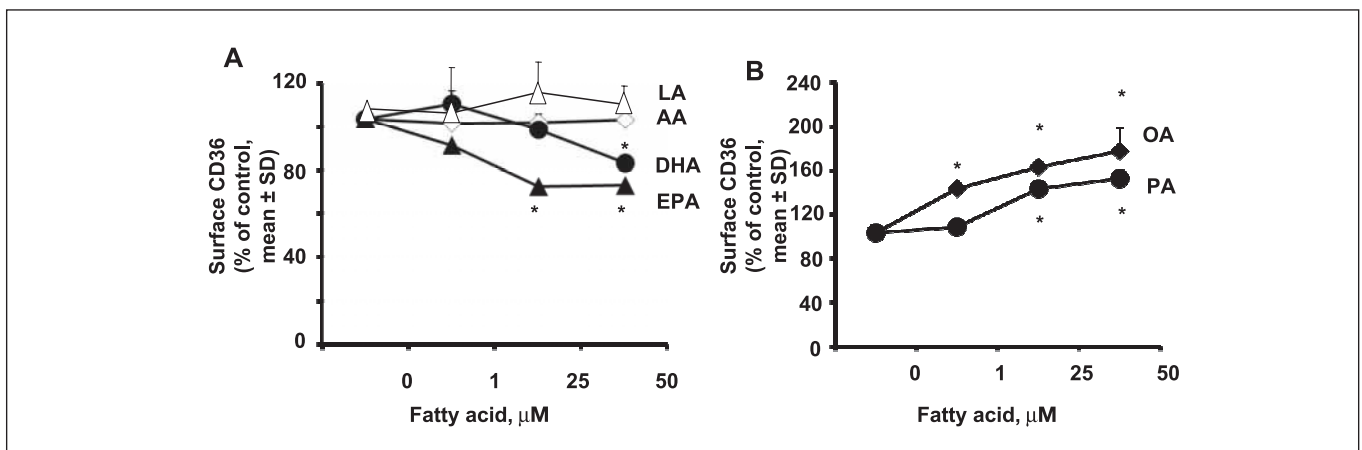
### Insulin, at pathophysiological concentrations, increases surface CD36 expression in HMVEC

Insulin induced a time- and concentration-dependent increase in CD36 surface expression. As shown in ►Figure 1A, the maximum increase in surface CD36 expression was observed after 24 h of incubation with insulin ( $10^{-8}$  M), and maintained for 24 h. To examine whether the effect was concentration-dependent, HMVEC were incubated for 24 h with various concentrations of insulin ( $10^{-9}$  –  $10^{-7}$  M). Insulin concentrations  $\geq 10^{-8}$  M induced a significant increase of CD36 protein expression, with a maximum increase seen at  $10^{-7}$  M (►Fig. 1B). The effects of insulin on CD36 expression was confirmed by flow-cytometry (►Table 1). No change was seen in total CD36 protein (at Western analysis) at any insulin concentrations, at any time point.



**Figure 1: Time- and dose-dependent effects of insulin on CD36 protein expression in HMVEC.** A, B) Human microvascular endothelial cells (HMVEC) were incubated for various times (0–72 h), or various concen-

trations of insulin (from 10<sup>-9</sup> to 10<sup>-7</sup> M); subsequently, cell surface expression of CD36 was measured by ELISA. Results are means ± SD of three separate experiments. P<0.05 vs. control at time 0 (A); P<0.01 vs. vehicle (B).



**Figure 2: The effect of various fatty acids on CD36 expression in HMVEC.** Human microvascular endothelial cells (HMVEC) were incubated for 48 h with palmitic acid (PA), oleic acid (OA), linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) (from

0 to 50 μM). At the end of incubations, cell surface expression of CD36 was measured by ELISA. Results are means ± SD of three separate experiments. \*P<0.05 vs. vehicle control.

### The effect of insulin on CD36 expression in HMVEC is mediated by the ERK1/2 MAPK insulin-signalling pathway

To explore the signalling pathway by which insulin increases CD36 protein expression, HMVEC were pretreated with inhibitors of PI3-kinase, p38 MAPK and ERK 1/2. As shown in ► Supplementary Figure 1 (available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)), in the absence of any cytotoxicity, pretreatment with the PI-3-kinase inhibitor wortmannin (at 100 nM) increased the effect of insulin on CD36 expression, while the ERK1/2 inhibitor PD98059 (at 50 μM) completely abolished this effect. The p38 MAPK inhibitor SB202190 (10<sup>-5</sup> M) did not show any effect on either the constitutive or the insulin-induced CD36 expression. These findings indicate that the activation of ERK1/2 – rather than of p38 MAPK – is required for the effect of insulin on CD36 protein expression in HMVEC. This effect is conversely enhanced by the simultaneous

inhibition of the metabolic PI-3-kinase-mediated pathway of insulin signal transduction.

### Various fatty acids differently regulate constitutive and insulin-induced CD36 expression in HMVEC

As CD36 is a high-affinity scavenger receptor for LCFA, we next determined the effects of several types of FA on the constitutive and insulin-induced CD36 expression in HMVEC. A ≥24 h exposure to the omega-3 FA EPA (at 25 and 50 μM) or DHA (at 50 μM) blunted both the constitutive (► Fig. 2A) and insulin-induced CD36 surface expression (► Fig. 3). We confirmed these effects by flow cytometry limited to DHA (► Table 1). Conversely, the omega-6 FA LA and AA (1–25–50 μM) did not alter constitutive or insulin-induced CD36 surface expression (► Figs. 2A and 3). When cells were treated with 25 and 50 μM palmitic acid or oleic

Treatment	% gated (FL1)	MFI (FL1)	% gated (FL2)	MFI (FL2)
Untreated	6 ± 0.1	177 ± 2	10 ± 0.1	202 ± 2
Insulin 10 <sup>-8</sup> M *	20 ± 8	358 ± 10	19 ± 9	268 ± 10
DHA 50 μM*	3 ± 4	132 ± 4	9 ± 4	140 ± 9
Insulin 10 <sup>-8</sup> M + DHA 50 μM**	6 ± 5	167 ± 8	15 ± 6	177 ± 10

Subconfluent HMVEC were pretreated with DHA for 12 h and/or insulin (10<sup>-8</sup> M) for further 24 h. After incubations, cells were exposed to Dil-oxLDL for 6 h, then immunostained for CD36 or FITC-conjugated IgG isotype control and submitted to flow-cytometry. Values are mean ± SD of % cells expressing CD36 (channel FL1) or uptaking oxLDL (channel FL2). Results are representative of three independent experiments. \* = P<0.05 vs. unstimulated control; \*\* = P<0.05 vs. insulin. MFI, mean fluorescence (FL) intensity.

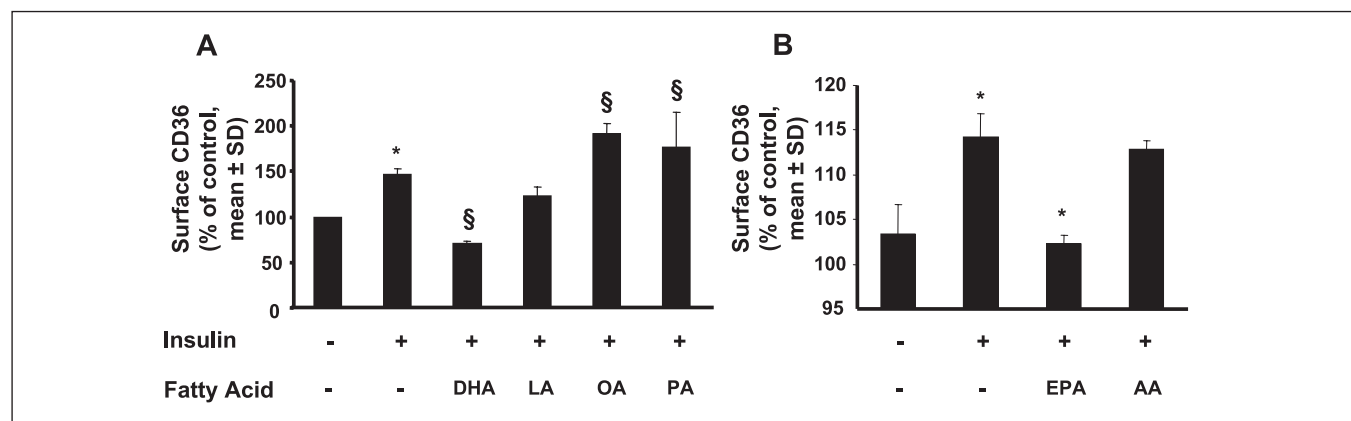
**Table 1: CD36 expression and oxLDL uptake by insulin and DHA at flow cytometry.**

acid, a saturated and a mono-unsaturated FA, respectively, a nearly two-fold increase in constitutive (► Fig. 2B) and insulin-induced (► Fig. 3) CD36 expression was observed compared with control conditions. None of the fatty acids tested affected the expression of total CD36 protein at Western analysis (data not shown).

### Various fatty acids differently regulate constitutive and insulin-induced uptake of oxLDL by CD36 in HMVEC

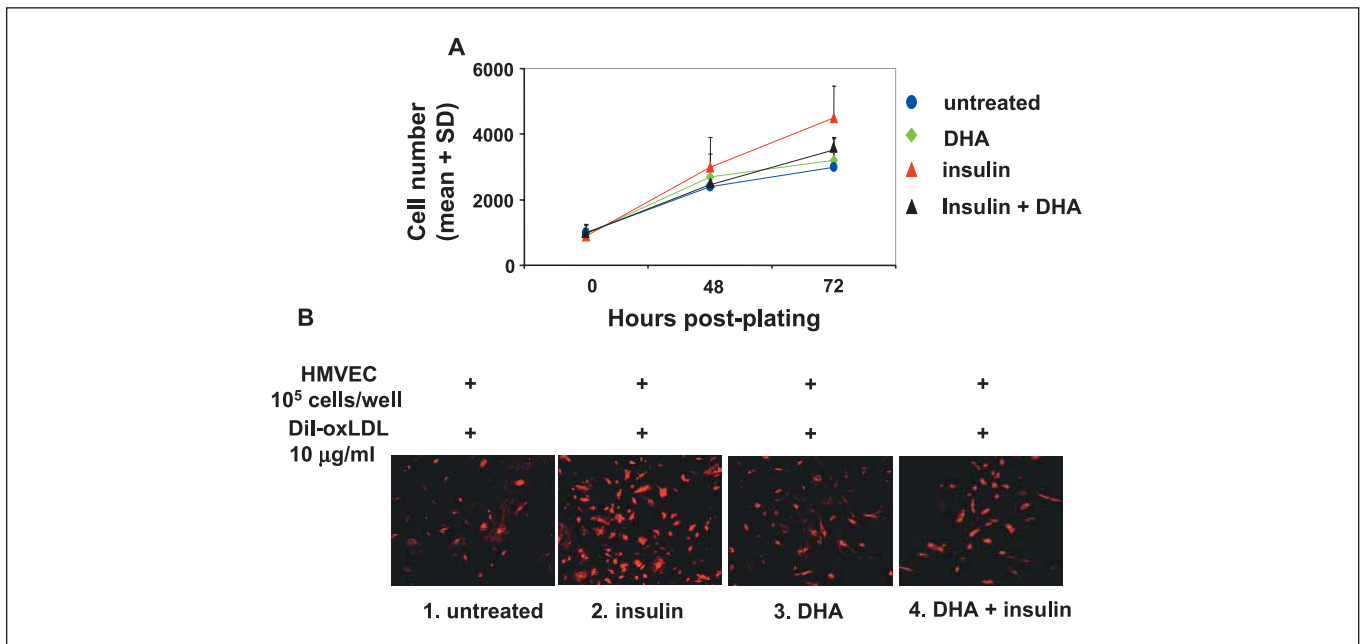
As CD36 is a high-affinity scavenger receptor for oxLDL, and as the effect of DHA on insulin-induced CD36 expression was more pronounced than for EPA, we next examined the effects of insulin and DHA on Dil-labelled oxLDL uptake in HMVEC. When cells were treated with insulin (10<sup>-8</sup> M), an increase in oxLDL uptake compared with untreated cells was appreciated by flow cytometry (► Table 1, ► Fig. 4 and ► Suppl. Fig. 2 available online at www.thrombosis-online.com). This was paralleled by an increase in CD36 surface expression. A substantial fraction of oxLDL uptake could be attributed to CD36 expression, since neutralising experi-

ments conducted by co-incubating HMVEC with insulin and a neutralising anti-CD36 antibody led to a marked (about 45%) decrease in ox-LDL uptake (► Table 2). Insulin also determined proliferative effects on HMVEC (► Fig. 4A). When cells were treated with insulin ± DHA (50 μg/ml), a decrease of both oxLDL uptake and surface CD36 expression was observed compared with cells treated with insulin alone, in the absence of any cytotoxic effect (► Table 1, ► Fig. 4 and ► Suppl. Fig. 2 available online at www.thrombosis-online.com). DHA also reverted insulin proliferative effects on HMVEC (► Fig. 4A). To further characterise the functional significance of insulin-induced uptake of ox-LDL in HMVEC, we measured the activity of endothelial nitric oxide synthase as a molecular marker of endothelial function, in terms of nitrite production and expression of active phosphorylated endothelial nitric oxide synthase (P-eNOS). As shown in ► Figure 5A, treatment of cells with high insulin decreased the expression of P-eNOS. A ≥24 h exposure to DHA (at 10–50 μM) *per se* increased the expression of P-eNOS and blunted the high insulin-induced reduction of P-eNOS (at 1–10–50 μM) (► Fig. 5A). DHA was, however, not able to revert the high insulin-induced suppression of P-eNOS in the presence of further inhibition by wortmannin of



**Figure 3: The effect of various fatty acids on insulin-induced CD36 expression in HMVEC.** Human microvascular endothelial cells (HMVEC) were preincubated for 12 h with palmitic acid (PA), oleic acid (OA), linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), all at 50 μM. After preincubation with FA, HMVEC were stimu-

lated for further 24 h adding insulin (10<sup>-8</sup> M final concentration) to FA-supplemented culture medium. At the end of incubations, cell surface expression of CD36 was measured by ELISA. Results are means ± SD of three separate experiments. \*P<0.05 vs. vehicle control; §P<0.05 vs. insulin treatment.



**Figure 4: The effect of insulin and DHA on proliferation and uptake of oxidised LDL in HMVEC.** Human microvascular endothelial cells (HMVEC) were pretreated with DHA for 12 h, then incubated with insulin for 48 h. A) Growth curves were acquired with untreated HMVEC (blue line) or insulin-and/or DHA-treated (red, green and black lines, respectively). Low-density-plated cells were counted at the indicated times. Photographs were taken two weeks after treatments. The number of HMVEC was determined by counting eight different high-power fields (h.p.f.) using an ocular grid and a

20 x objective (0.09 mm<sup>2</sup>/field). Fields for counting HMVEC were randomly located at half-radius distance from the centre of the monolayers. Results are mean  $\pm$  SD of counted cells, from n=3 experiments. B) Representative photomicrographs of HMVEC treated with insulin and/or DHA and then exposed to Dil-oxLDL for 6 h at 37°C, indicating the uptake of oxLDL in untreated (panel 1) vs. treated cells (panel 2, with insulin; panel 3, with DHA; panel 4, with insulin + DHA). Red fluorescence: cells positive for Dil-oxLDL uptake.

phosphoinositide-3 (PI3)-kinase-mediated insulin pathway (► Fig. 5A). Conversely, DHA and insulin did not alter total CD36 expression (► Fig. 5B). In parallel with P-eNOS expression, high insulin decreased nitrite production (► Fig. 5C), suggesting endothelial dysfunction, which was reverted by a  $\geq 24$  h exposure to DHA (at 1–10–50  $\mu$ M). Neutralising experiments to test the CD36-dependence of oxLDL uptake showed a significant, albeit minor, increase of nitrite production in HMVEC treated with insulin in such conditions, suggesting a role of oxLDL uptake via CD36 in determining high insulin-induced endothelial dysfunction (► Fig. 5D).

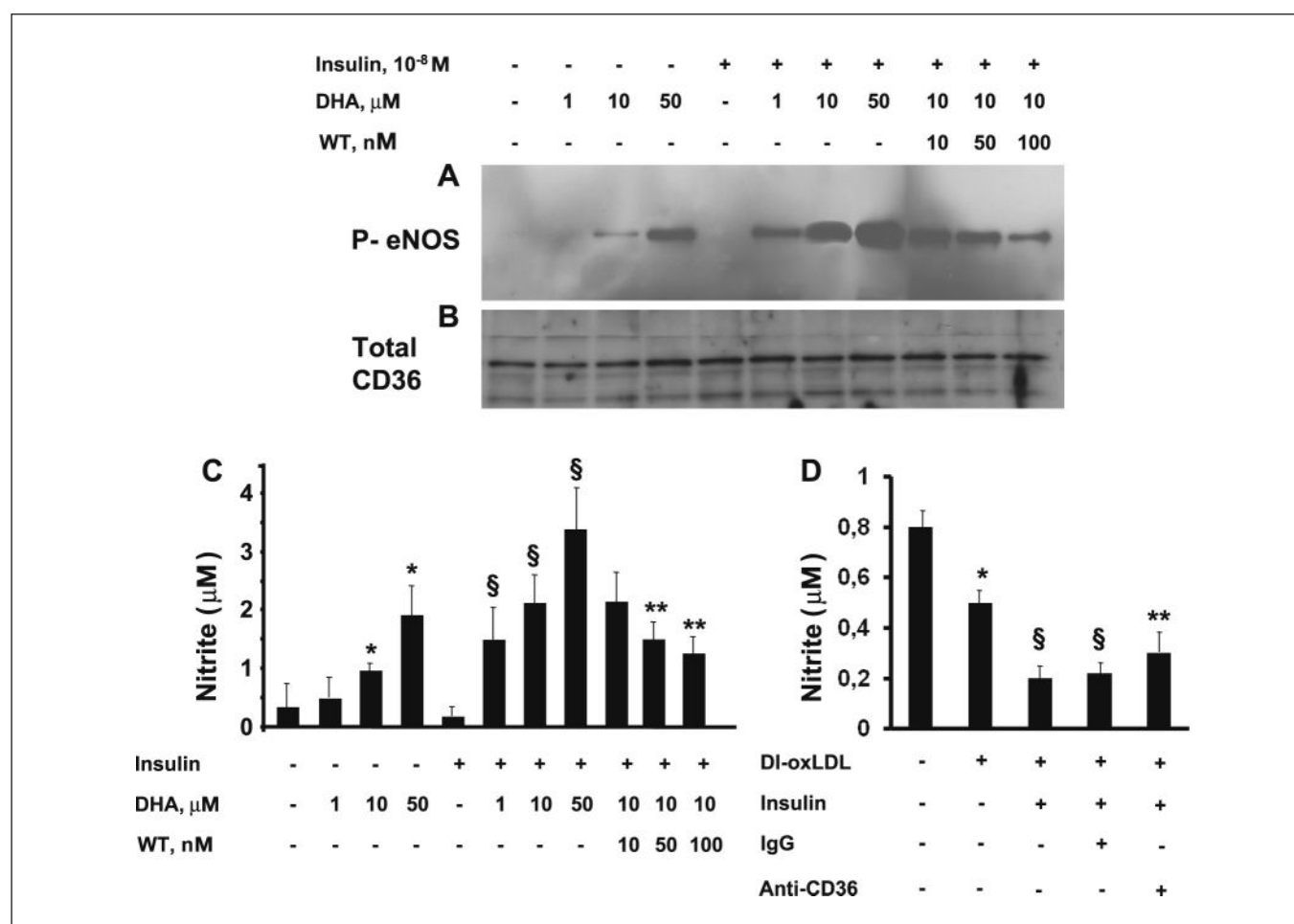
### The effect of DHA on insulin-induced CD36 expression is mediated by suppression of peroxisome PPAR $\alpha/\gamma$

CD36 is known to be one of the PPAR target genes, the expression of which is regulated by PPAR $\alpha/\gamma$  activation in monocytes, macrophage and microvascular endothelial cells (20). DHA is known to bind and suppress PPAR activation in some cell types (21). We therefore determined whether the modulation of CD36 induced by DHA in insulin-treated HMVEC could be a consequence of PPAR $\alpha/\gamma$  suppression. HMVEC were pretreated with DHA (10–50

**Table 2: CD36-dependence of oxLDL uptake in microvascular endothelial cells at baseline and after exposure to high insulin levels.**

Treatment	Cell number/well (RFU 350ex-470em)	OxLDL uptake/cell number/well (RFU, 530ex-590em/RFU 350ex-470em)
Baseline	5527 $\pm$ 774	0.28 $\pm$ 0.01
Insulin 10 <sup>-8</sup> M	14755 $\pm$ 2838	0.99 $\pm$ 0.1 *
Insulin 10 <sup>-8</sup> M + control IgG	14889 $\pm$ 1592	0.87 $\pm$ 0.2
Insulin 10 <sup>-8</sup> M + anti-CD36 antibody	7995 $\pm$ 987	0.55 $\pm$ 0.02 §

Subconfluent human microvascular endothelial cells were pretreated with insulin (10<sup>-8</sup> M) for 24 h, in the presence or absence of a neutralizing anti-CD36 antibody or isotype control IgG. After incubation, cells were exposed to Dil-oxLDL for 6 h, then stained with DAPI and submitted to microplate spectrofluorimetry. Values are mean  $\pm$  SD of relative fluorescence units (RFU) for Di-oxLDL uptake (RFU-oxLDL), after normalisation for cell number (RFU cell number per well), setting excitation (ex) and emission (em) wavelength at 530 and 590 nm, respectively, for Di-oxLDL, and at 350 and 470 nm, respectively, for cell number. Results are representative of three independent experiments. \* = P<0.05 vs. unstimulated control; \*\* = P<0.05 vs. insulin.



**Figure 5: The effect of insulin and DHA on nitrite production and expression of phosphorylated endothelial nitric oxide synthase in HMVEC.** Human microvascular endothelial cells (HMVEC) were pretreated with DHA for 12 h, then incubated with high insulin (10<sup>-8</sup> M) for 48 h. Subsequently, the expression of phosphorylated endothelial nitric oxide synthase (A), total CD36 (B), and nitrite production (C) in the culture medium were assessed. Filters incubated with anti-CD36 antibody were stripped and

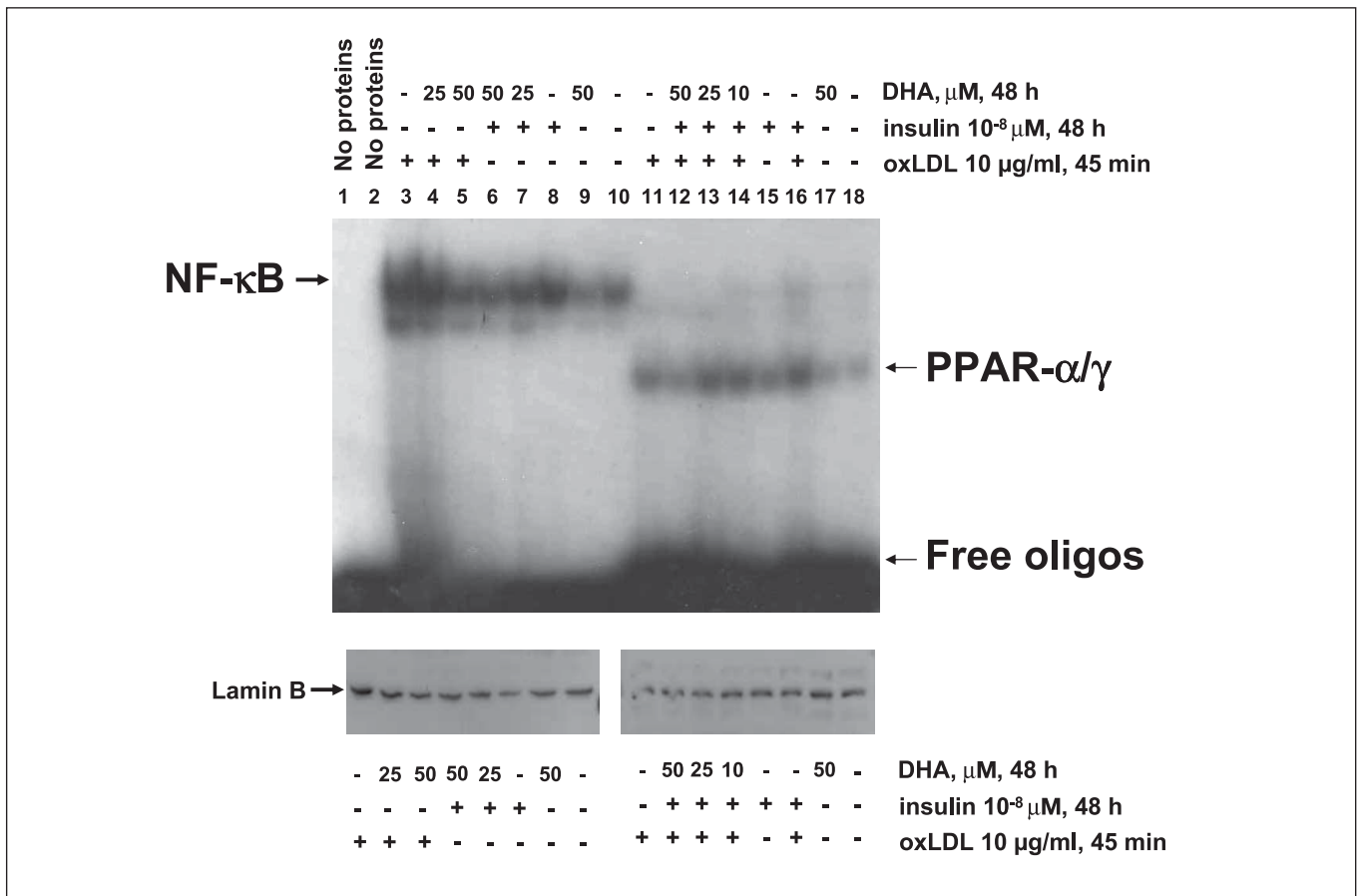
reprobed with an anti-P-eNOS antibody. Results are means ± SD of three separate experiments. \*P<0.05 vs. vehicle control; § P<0.05 vs. insulin; \*\*P<0.05 vs. insulin+DHA. D) Nitrite production in HMVEC pre-incubated with high insulin (10<sup>-8</sup> M) for 48 h in the presence or absence of a neutralising anti-CD36 antibody, and subsequently treated with DI-oxLDL for 6 h. Results are means ± SD of three separate experiments. \*P<0.01 vs. vehicle control; § P<0.01 vs. DI-oxLDL; \*\*P<0.05 vs. insulin+DI-oxLDL.

μg/ml) for 12 h and then co-incubated with insulin (10<sup>-8</sup> M) for 48 h (► Fig. 6). Forty-five minutes before collecting nuclear proteins, cells were exposed to oxLDL (10 μg/ml). We observed a strong increase of DNA binding of PPARα/γ in cells treated with insulin and/or oxLDL (► Fig. 6, lanes 11, 15, and 16), which was partially suppressed by DHA in a concentration-dependent manner (► Fig. 6). In the same experimental conditions, oxLDL or insulin markedly increased the activation of NF-κB (► Fig. 6, lanes 3 and 8), which was partially reduced by DHA in a concentration-dependent manner (► Fig. 6). There was no significant and specific binding with the unlabelled (cold) oligonucleotide, or by omitting the nuclear protein extracts (► Fig. 6, lane 1 and 2) or the oligonucleotide probe (not shown), as well as by using nuclear protein extracts from unstimulated HMVEC (► Fig. 6, lane 10 and 18). Consistently, immunoblotting demonstrated that HMVEC treated with insulin for 48 h showed an increased nuclear accumulation of

NF-κB p65 and p50 subunits compared with untreated cells (see ► Suppl. Fig. 3 available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)). The presence of both NF-κB subunits p50 and p65 in untreated cells was barely detectable (see ► Suppl. Fig. 3 available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)). We also observed an at-least-additive effect on TNF-α-induced nuclear accumulation of p65 and p50 subunits by adding insulin (10<sup>-8</sup> M) to TNF-α (see ► Suppl. Fig. 3 available online at [www.thrombosis-online.com](http://www.thrombosis-online.com)). Thus the expression of NF-κB subunits correlates with the presence of NF-κB activity in the nuclear extracts. Therefore, treatment with insulin was here shown to promote NF-κB nuclear accumulation, thus accounting for the NF-κB translocation seen by EMSA.

To further investigate the involvement of PPARs in the effect of DHA on insulin-induced CD36 expression, HMVEC were pretreated with a PPARα/γ antagonist GW9662 (5 μM) or DHA for 12 h before incubating cells with insulin. Co-treatment of HMVEC





**Figure 6:** The effect of DHA on PPAR $\alpha/\gamma$  and NF- $\kappa\text{B}$  activation in insulin- or oxLDL-treated HMVEC. Electrophoretic mobility gel shift assay (EMSA), showing the effect of DHA  $\pm$  insulin and/or oxLDL on PPAR $\alpha/\gamma$  and NF- $\kappa\text{B}$  activations. EMSA was performed by mixing the  $^{32}\text{P}$ -oligonucleotide encoding for the PPAR $\alpha/\gamma$  or NF- $\kappa\text{B}$  consensus sequence with nuclear proteins from human microvascular endothelial cells (HMVEC) treated with insulin ( $10^{-8}$  M) and/or DHA (10–25–50  $\mu\text{M}$ ) for 48 h before oxLDL stimulation

(10 ng/ml), added to HMVEC during the last 45 min of insulin and DHA treatments. Results are representative of three separate experiments. No significant binding was observed with the unlabelled (cold) oligonucleotide, or by omitting the nuclear protein extracts (lanes 1 and 2) or the oligonucleotide probe (not shown), as well as by using nuclear protein extracts from unstimulated HMVEC. The equal loading of nuclear proteins was verified by Western analysis using an antibody against lamin B.

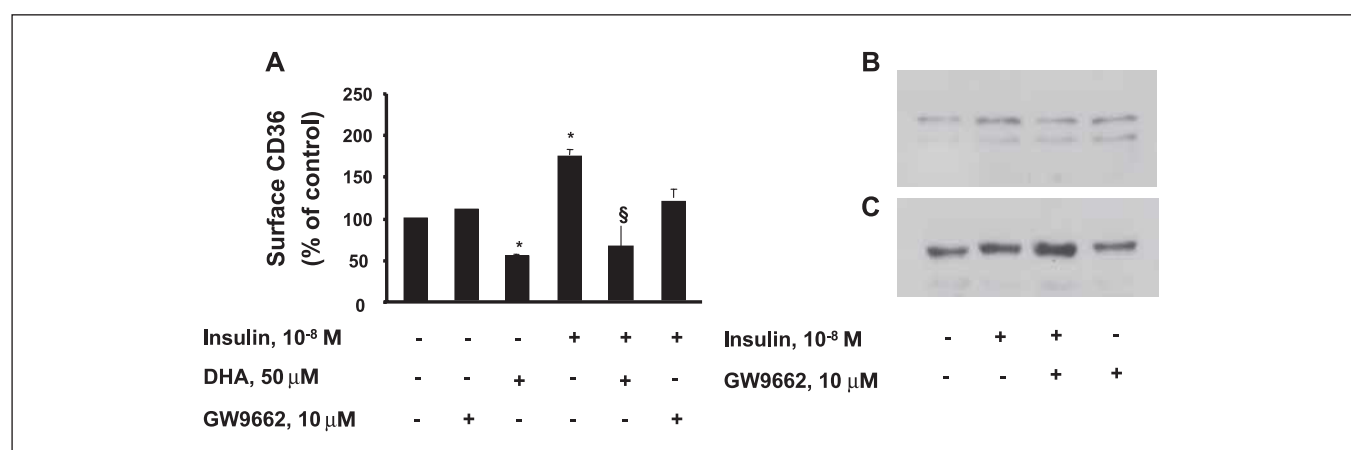
with insulin and GW9662 completely reverted CD36 expression induced by insulin. DHA mimicked this effect (► Fig. 7A). Treatment of HMVEC with insulin increased the plasma membrane content of CD36 (► Fig. 7B), while co-treatment of HMVEC with insulin and GW9662 shifted the expression of CD36 back into the cytoplasm (► Fig. 7C), indicating that PPAR $\alpha/\gamma$  influences CD36 expression by acting on its intracellular trafficking. These data overall demonstrate that insulin induces CD36 expression by PPAR $\alpha/\gamma$  activation, and DHA likely suppresses insulin stimulation through a similar mechanism.

## Discussion

We had previously demonstrated that insulin, at pathophysiologically relevant concentrations, exerts pro-inflammatory, pro-atherogenic effects on macrovascular endothelial cells (human aortic

endothelial cells and human umbilical vein endothelial cells) by enhancing vascular cell adhesion molecule (VCAM)-1 expression and monocyte-endothelial interactions (22–25). The present findings (a) demonstrate the ability of insulin to act also on microvascular endothelial cells, alone or in concert with saturated FA (such as palmitic acid), or n-9 monounsaturated fatty acids (such as oleic acid) in enhancing surface expression of CD36; (b) demonstrate the ability of the n-3 polyunsaturated FA EPA and DHA to decrease insulin-induced CD36 expression; (c) provide insights on the underlying signalling pathway. This is – to the best of our knowledge – the first report showing additive effects of insulin and saturated/monounsaturated FA and opposite effects of n-3 polyunsaturated FA on CD36 expression on microvascular endothelial cells.

We used insulin concentrations between  $10^{-9}$  and  $10^{-7}$  M, in the range of binding and activation of insulin receptor in endothelial cells (26). Such concentrations, equivalent to 139–13,900  $\mu\text{U/ml}$ , span from pathophysiological to pharmacological levels of insulin



**Figure 7: The effect of the PPAR $\gamma$  antagonist GW9662 and DHA on insulin-induced CD36 expression in HMVEC.** Human microvascular endothelial cells (HMVEC) were preincubated for 12 h with GW9662 (10  $\mu$ g/ml) or DHA (50  $\mu$ M), and then incubated with insulin (10<sup>-8</sup> M) for further 24 h. A) Cell surface expression of CD36 was measured by ELISA. Results are

means  $\pm$  SD of three separate experiments. \* $P < 0.05$  vs. vehicle control; § $P < 0.05$  vs. insulin. B) Plasma membrane and C) cytoplasmic expression of CD36, assessed by Western analysis. Blots are representative of three independent experiments.

in the blood. Concentrations of 10<sup>-9</sup> and 10<sup>-8</sup> M are indeed commonly seen in fasting and post-prandial states of individuals with insulin resistance (27), indicating the plausibility that these *in vitro* observations are applicable to *in vivo* settings where hyperinsulinaemia occurs. Concentrations of FA used in these experiments ( $\leq 50$  M) are also compatible with, or slightly higher than, *in vivo* dietary or pharmacological supplementation, shown to be in the micromolar range (28). Also, the time course of the inhibitory effects of DHA and EPA and the additive effects of oleic acid and palmitic acid are fully compatible with that of FA incorporation into endothelial cell membranes, previously shown to plateau after 48 h (29).

Our results demonstrate that – contrary to saturated FA such as palmitic acid, or n-9 monounsaturated FA such as oleic acid – DHA and EPA, the major n-3 polyunsaturated FA present in marine lipids, significantly inhibit both the constitutive and insulin-induced CD36 expression in HMVEC. Polyunsaturated FA and their oxidation products are reported to modulate CD36 gene expression in human macrophages. The omega-3 FA EPA and DHA are thought to protect against cardiovascular diseases. However, mechanisms by which fish oils may confer their benefits are not fully understood. CD36 is known to be one of the PPAR target genes, the expression of which is regulated by PPAR $\alpha/\gamma$  in monocytes and macrophages (20). Forman et al., using a luciferase reporter gene assay with plasmid containing the binding sites for PPAR $\alpha/\gamma$  (PPRE), reported that DHA, contrarily to LA, induced strong transcriptional activation of PPAR $\delta$  in contrast to its weak ligand-binding activity to PPAR $\gamma$  (30). It has also been reported that n-3 polyunsaturated FA such as EPA,  $\alpha$ -LA, or DHA, suppress the basal transactivating activity of PPAR $\gamma$ , whereas n-6 polyunsaturated FA such as LA and AA, and n-9 monounsaturated FA such as OA, stimulate such activity in human mammary gland carcinoma cells (MCF-7 cells) (31).

Our results indicate that CD36 expression is suppressed by DHA and EPA in HMVEC. Furthermore, our results demonstrate that insulin induces CD36 expression by PPAR $\alpha/\gamma$  activation, and that DHA reduces this expression by suppressing PPAR $\gamma$  activation. These observations therefore confirm previous work by other laboratories showing that EPA and DHA reduce CD36 expression at both mRNA and protein levels in U937 human monocytoid cells, with no effect for LA (11). However, contrary to our current findings, treatment with EPA, DHA and, to a lesser extent, OA and LA had been reported to significantly enhance mRNA and protein levels of CD36 in THP-1 cells and monocyte-derived macrophages (32). Such differences may depend on the different cell types used in these different reports. Previous study suggested that n-3 FA suppress tumour cell growth (33). In our study we observed an antiproliferative effect of DHA on HMVEC proliferation at baseline and after high insulin treatment. PPARs are known to regulate genes involved cell proliferation, differentiation and apoptosis (34). Several reports have shown that PPARs are linked to colon cancer. Suppression of PPAR $\gamma$  activation may therefore represent a mechanism by which n-3 FA exert an antiproliferative effect. By showing the ability of insulin to induce the activation of PPAR $\alpha/\gamma$ , we here propose the existence of a cross-talk between the PPAR $\alpha/\gamma$  and insulin signalling pathways, by which the activation of ERK1/2 MAPK by insulin is most likely responsible for the activation of the PPAR $\alpha/\gamma$  transcriptional activity, perhaps through PPAR phosphorylation. A number of studies have focused on the regulation of PPAR $\alpha/\gamma$  by insulin and FA, with contrasting results. Some authors have found that activation of MAPK induced inhibited PPAR $\alpha/\gamma$  transcriptional activity (35, 36), while others have shown the capacity of insulin- and MAPK-mediated phosphorylation to induce PPAR $\alpha/\gamma$  activation (37, 38). One reason for the discrepancy between our current findings and some of previous data in the literature may be that most previous experiments were performed on fibroblasts ectopically overexpressing

PPAR $\alpha/\gamma$ . Again, differences may therefore depend on the different cell types used, consistent with the notion that the expressions of such signalling molecules appears to differ between fibroblasts and endothelial cells, resulting in different molecular events. Alternatively, insulin upregulates the expression of genes involved in FA synthesis and LDL internalisation via induction of sterol-regulatory-elements-binding-protein 1c (SREBP-1c) promoter (39). This involves the combined action of several transcription factors, including liver X receptor (LXR) $\alpha$ , Sp1 and nuclear factor Y (NF-Y). Specifically, insulin increases the activity of LXR $\alpha$  by 1) increasing concentration of the endogenous LXR $\alpha$  agonist; and 2) by promoting covalent modification of LXR $\alpha$  (39). Also, insulin increases the phosphorylation of Sp1 and its O-Glc-NAc modification, promoting its nuclear translocation and, thereby, interaction with SREBP-1c (39). Conversely, n-3 FA decrease lipogenic gene transcription via suppression of SREBP-1c, suggesting a further mechanism of antagonism between DHA and insulin (40).

In the current study, we could also observe a decrease of insulin-induced cell proliferation in HMVEC co-incubated with a neutralising anti-CD36 antibody. The mechanism responsible for the downregulated HMVEC proliferation has not been explored. It might occur due to blocking of CD36-dependent oxLDL uptake, which is a known proliferative stimulus for different cell types, including endothelial cells, or some other mechanism.

Hyperinsulinaemia mimicked by our *in vitro* study, is a hallmark of insulin resistance, promoting inflammation and endothelial dysfunction. Very recently it has been shown that n-3 FA (DHA and EPA) exert insulin-sensitising and anti-inflammatory effects in macrophages and adipocytes by signalling through G-protein-coupled receptor 120 (GPR120) (41). Researchers found, in wild-type C57 mice but not in GPR129 knockout mice, that n-3 FA supplementation markedly increased insulin sensitivity in terms of glucose uptake, along with decreased levels of insulin. In parallel, several markers of systemic inflammation [inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-6] were decreased. Taken together, these data sug-

gest a further mechanism by which n-3 FA counteract detrimental effects of insulin resistance/hyperinsulinaemia.

In our experimental conditions in HMVEC we also showed the ability of oxLDL and insulin, alone or in combination with low concentrations of TNF- $\alpha$ , to activate also the transcription factor NF- $\kappa$ B, traditionally implicated in the expression of proinflammatory cytokines and endothelial adhesion molecules, such as VCAM-1, intercellular adhesion molecule (ICAM)-1 and E-selectin. This might contribute to amplify the induction of CD36 in HMVEC by a paracrine mechanism.

In conclusion, by mimicking hyperinsulinaemia and the simultaneous exposure of cells to high concentrations of FA, such as occurring in conditions of insulin resistance, we here show that insulin, acting through the ERK1/2 mitogenic signalling pathway and PPAR $\alpha/\gamma$  activation, increases CD36 protein expression in HMVEC, and that these effects are potentiated by saturated or monounsaturated n-9 FA, while DHA and – to a lesser extent – EPA, decrease such stimulatory effects. These phenomena may be involved in the microvascular endothelial dysfunction and the progression of microvascular disease occurring in patients with the metabolic syndrome and type 2 diabetes. Further characterisation of the mechanisms involved may lead to the development of novel therapeutic strategies to prevent or reverse the progression of microangiopathy in such conditions.

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#### Conflict of interest

None declared.

#### What is known about this topic?

- CD36 is a PPAR $\alpha/\gamma$ -regulated scavenger receptor and long-chain free fatty acid transporter implicated in myocardial dysfunction in type 2 diabetes and insulin resistance.
- Its role in endothelial cells is largely unknown.
- An enhanced uptake of free fatty acids (FFA) and oxidised low-density lipoproteins (oxLDL) by endothelial cells may lead to oxidative stress and microvascular dysfunction.

#### What does this paper add?

- We here document that CD36 surface expression in microvascular endothelial cells is time- and concentration-dependently increased by insulin, and such expression is specifically attenuated by the omega-3 fatty acids DHA and EPA.
- Such mechanisms may be the basis for the use of omega-3 fatty acids in diabetic microvasculopathy.

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