The putative diabetic plasma marker, soluble CD36, is non-cleaved, non-soluble and entirely associated with microparticles

M. J. ALKHATATBEH,*§ N. M. MHAIDAT, ‡ A. K. ENJETI, †§ L. F. LINCZ †§ and R. F. THORNE*§

** Cancer Research Unit, School of Biomedical Sciences and Pharmacy, Faculty of Health, the University of Newcastle, NSW, 2308; †Hunter Haematology Research Group, Calvary Mater Newcastle Hospital, Waratah, NSW, 2298; § Hunter Medical Research Institute, Australia; and ‡ Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan.*

Address correspondence to:

Rick F. Thorne
Cancer Research Unit
School of Biomedical Sciences & Pharmacy
The University of Newcastle
NSW 2308, Australia
Tel. +61-2-49217860
Fax. +61-2-49216903
E-mail: Rick.Thorne@newcastle.edu.au

Lisa Lincz,
Hunter Haematology Research Group
Calvary Mater Newcastle
Edith Street, Waratah
NSW 2298, Australia
Tel. + 61-2 49211844
Fax. +61-2 49602136
E-mail: Lisa.Lincz@newcastle.edu.au

Keywords: atherosclerosis; insulin resistance; lipoproteins; microparticles; scavenger receptor;

soluble CD36

Summary (250 words)

Background: CD36 is a widely expressed cell surface receptor that binds lipoproteins and its function has been implicated in many complications of the metabolic syndrome. A cell-free form of CD36, soluble CD36 (sCD36), has been reported in human plasma and found to be elevated in obesity and diabetes and claimed as a marker of insulin resistance.

Objective: To determine the nature of sCD36; in particular whether sCD36 is truly soluble or, as hypothesized, is found as a component of circulating microparticles (MPs).

Methods: Lipoproteins were fractionated by density gradient centrifugation, plasma MPs were isolated by ultracentrifugation, size exclusion and immunoprecipitation with CD36 detected by immunoblotting. MPs from plasma and activated platelets were analysed by multi-colour flow cytometry using a DyLight-488 anti-CD36-conjugate in combination with antibodies against different cellular markers.

Results: cell-free plasma CD36 was not observed associated with lipoproteins and was not a proteolytic fragment; rather it was associated with the plasma MP fraction suggesting that sCD36 in the plasma of normal subjects is a product of circulating MPs. Cytometric and immunoblotting analyses of plasma from normal donors showed these MPs were derived mainly from platelets. Analysis of *in vitro* activated platelets also showed that CD36 to be secreted in the form of MPs.

Conclusions: sCD36 is not a proteolytic product but rather is associated with a specific subset of circulating MPs that can readily be analysed. This finding will enable more specific investigations into the cellular source of the increased levels of plasma CD36 found in subjects with diabetes.

(Manuscript, **4529** words excluding references)

Introduction

CD36 is a transmembrane glycoprotein originally identified in platelets [1, 2], and subsequently found to be expressed on a wide range of other cells and tissues [3-6]. CD36 is a heavily N-glycosylated membrane protein [7] that was characterized as the most proteolytically resistant protein of the platelet membrane [1]. It has two intracellular domains, two transmembrane domains and a large extracellular domain containing ten N-linked glycosylation sites which contribute to variations in its molecular weight [8, 9]. The intracellular domain consists of two very short cytoplasmic tails that are characterized by pairs of palmitoylated cysteine residues [10].

CD36 is a multifunctional signaling molecule with several known ligands. These include thrombospondin-1 [11], long chain fatty acids [4], the native lipoproteins HDL, LDL, and VLDL [12], and modified lipoproteins including oxidized LDL [3]. Various studies have implicated CD36 involvement in lipid metabolism and angiogenesis, and in the pathological conditions of atherosclerosis, stroke, Alzheimer's disease, cancer biology, inflammation, insulin resistance, diabetes and diabetic nephropathy [5, 13, 14].

Recently, Handberg et al [15] demonstrated the presence of a soluble circulating form of CD36 termed sCD36 that was detected in human plasma by ELISA. They concluded that sCD36 was highly associated with insulin resistance, a risk factor for accelerated atherosclerosis in type 2 diabetes, and proposed that sCD36 could be used as a marker of the metabolic syndrome and atherosclerosis [15]. Further analysis showed that sCD36 levels cluster with markers of insulin

resistance and elevated sCD36 was correlated with increased risk of type 2 diabetes [16]. They also found that sCD36 could be a marker of atherosclerotic plaque instability and considered this important since the risk of cardiovascular disease is associated with composition and stability of the atherosclerotic plaque rather than the degree of the stenosis in arteries [17]. Further, sCD36 correlated with liver injury that is associated with insulin resistance in patients with altered glucose tolerance [18].

The nature and origin of the sCD36 has not been determined, although it has been postulated to be either a product of proteolytic cleavage or as an intact glycoprotein in the form of circulating microparticles (MPs). Some authors have built on the idea that sCD36 represents the CD36 ectodomain [19, 20], and Wang et al extrapolated this concept to propose that a recombinant human sCD36 extracellular domain could be used as a CD36 antagonist to target atherosclerosis at the level of CD36-ligand binding [20].

Knowledge about the nature of sCD36 is important for a number of reasons. First, CD36 on platelets was initially characterized as being highly resistant to proteolysis [1]. Therefore, a proteolytic product comprising only the ectodomain might represent a novel type of cleavage by cells of unknown origin or a hitherto unknown secreted isoform of CD36. However, our hypothesis is that sCD36 is not a proteolytic cleaved product but is in fact a component of a sub-group of MPs. If this can be established, the readily available methods for characterizing MPs will enable further studies to determine the cellular source of the increased sCD36 found in subjects with diabetes. Thus the aim of the present study was to characterize sCD36 found in human plasma.

Materials and Methods

Antibodies and Immunoblotting

The polyclonal antibody platelet glycoprotein IIb (CD41) was a gift of D. Pidard (Unite´ de Pharmacologie Cellulaire, Unite´ Associe´e IP/INSERM 285, Institut Pasteur, Paris). Mouse monoclonal antibodies (mAb) against CD36, Long9 (used for immunoblotting) and 11H5 (used for immunoprecipitation, flow cytometry and ELISA) and blotting antibodies against PTA1 (NEW E1) together with the immunoblotting method used (Enhanced Chemiluminescence) were as previously described [21, 22]. Affinity purified rabbit anti-CD36 polyclonal antibodies were as previously described [23].

Blood collection

Biochemical experiments were conducted using blood samples from healthy volunteers. Fresh whole blood was collected by venepuncture using 21 gauge needles. Each sample was transferred to a centrifuge tube containing 2mM as a final concentration of Ethylenediaminetetraacetic acid (EDTA) as anticoagulant and was processed immediately.

Lipoprotein fractionation

Whole blood was centrifuged at 400g for 20min at room temperature and the platelet rich plasma (PRP) collected. Residual cells and platelets were then removed by further sequential centrifugation steps, 800g for 10min, 3000g for 20min, and finally 100,000g for 15min at 15°C in an SW41 ultracentrifuge rotor (Beckman, IL, USA). Clarified plasma was collected and diluted 4:1 in Optiprep media (Axis-Shield, Artarmon, Australia) and the samples sealed in Optiseal polyallomer tubes and ultracentrifuged in a Type 70.1 rotor (Beckman) at 250,000g for 16h at 15°C. Tubes were fractioned into 16 equal fractions by tube puncture and equal volumes

were applied to 0.7% agarose gels made and electrophoresed in 50mM Tris-HCl pH 7.4 buffer. Staining of lipoproteins was performed using 1 % (w/v) Sudan Black in 60% ethanol. Aliquots of the same fractions were subjected to immunoblotting.

Isolation and activation of platelets

Platelet rich plasma (PRP) was obtained from whole blood centrifuged at 400g for 5min at room temperature. Washed platelets were prepared by centrifugation of PRP at 1500g for 15min and the platelets further washed and resuspended in Tyrode's buffer at a concentration of 200 x 10^6/ml. The platelet agonists PMA (100nM; Sigma) and thrombin (1 U/ml murine thrombin) were added in the presence of Pefabloc (1:20 dilution; Roche). Low speed pellets and supernatant fractions were prepared by centrifugation of the samples at 3000g for 20min.

Preparation of plasma MPs and platelet MPs by ultracentrifugation

Platelet free plasma (PFP) was prepared by serial double centrifugation of fresh blood samples at 3,000g for 15 min at room temperature. PFP was then ultracentrifuged for 1 hr at 4°C at 100,000g in an SW55 rotor (Beckman). Following ultracentrifugation, the supernatant was removed carefully and the pellet washed twice and resuspended in a small volume of phosphate buffered saline (PBS), pH 7.4 prior to further analysis. Platelet MPs were similarly prepared from the low speed supernatants obtained as described in the preceding section.

Isolation of plasma MPs by size exclusion

PFP was applied to a 120 x 2.4 cm Sepharose® CL-4B size exclusion column (Pharmacia) connected to a BioLogic liquid chromatography system (BioRad). The mobile phase

was PBS, pH 7.4 and the flow rate was 1 ml/minute. 5 ml PFP was applied to the column and 40 fractions of 5 ml each were collected. The protein concentrations of the fractions were determined using Micro BCA™ Protein Assay Reagent Kit (Thermo Scientific). Void volume fractions were pooled and ultracentrifuged for 1 hr at 4°C at 100,000g in an SW32 rotor (Beckman). Then, the supernatant was aspirated carefully and the pellet to be analyzed was resuspended in a small volume of PBS.

Isolation of circulating CD36 by immunoprecipitation

CD36 was immunoisolated from PFP using PureProteome™ Protein G Magnetic Beads (Millipore). Briefly, PFP samples were incubated with anti-CD36 antibody 11H5- conjugated protein G magnetic beads for at least 3 hours at 4°C with continuous mixing by rotation. Bound material was isolated using a strong magnet and further washed using 0.1 M Tween 20 (v/v) in PBS. Samples were eluted from the beads using lithium dodecyl sulfate loading buffer (LDS; Invitrogen) and heated at 70°C for 10 minutes before immunoblotting.

Analysis of MPs by flow cytometry

Staining and analysis of MPs was performed essentially as previously described [24] and according to guidelines established by the International Society on Thrombosis and Haemostasis Vascular Biology SSC on the Standardization of FMC-based PMP enumeration by flow cytometry [25] incorporating modifications suggested for the BD FACS Canto (BD Biosciences, San Jose, CA, USA). To calibrate the cytometer, a blend of 2:1:1 0.5, 0.9 and 3 μm diameter fluorescent beads (Megamix, Monocytex) was used to ensure adequate FSC resolution and set the lower MPs detection limit according to the manufacturer's instructions. This was achieved by adjusting the FSC threshold to allow detection of approximately 50% of the 0.5 μm beads,

corresponding to the median level and therefore the nominal diameter of the beads. The upper limit of detection is ordinarily set around the 0.9 µm bead cloud to focus on MPs in a defined size range of 0.5-1 µm.

MPs were detected in PFP by triple fluorescence flow cytometry using an antibody against CD36 (clone 11H5) conjugated to DyLight-488 (Thermo Scientific) in various combinations with CD41-PE (clone PL2-49, Biocytex), annexin V-APC (eBioscience), CD14-PE (clone TÜK4, MACS miltenyl), and CD62e-PE (clone 68-5H11, BD Pharmingen). All samples were prepared in Calcium rich annexin V binding buffer (eBiosciences Inc.) and analyzed on a BD FACS Canto (BD Biosciences). Events were collected for 60 seconds at low flow rates for each sample.

ELISA assay

ELISA plates (Microlon high-binding; Greiner bio one) were pre-coated with anti-CD36 mAb (11H5; 500 ng/well) overnight at 4°C before blocking using 5 % w/v skim milk in PBS buffer containing 0.05% v/v Tween-20. Samples were applied for 2 h at RT and following this rabbit polyclonal antibodies against CD36 or CD41 were added for 2 h at RT. Immunocomplexes were detected using anti-rabbit IgG-HRP-conjugate (1:5000 (v/v); BioRad) and quantitated using ABTS substrate solution (0.9mM ABTS and 2.65 mM H_2O_2 in citrate buffer, pH 5.5). After development, absorbance readings were taken at 405 nm using a SpectraMAX microplate reader (Molecular Devices). All samples analysed were diluted in PBS and all washes performed using PBS-Tween-20 buffer. Affinity isolated platelet CD36 protein was as previously described [23].

Results

Consistent with previous reports where sCD36 had been detected in plasma [15], we were able to detect CD36 reactivity in whole human PFP using an ELISA assay (Supplementary Fig.1A and B). Similarly, Western blotting of PFP from a number of subjects (n=6) consistently detected low levels of sCD36 using a CD36-specific monoclonal antibody (Long 9) that is highly sensitive in Western blotting applications (data not shown). Thus, although Western blotting detection of CD36 detection was inefficient, largely because the amount of plasma that can be analyzed by electrophoresis is limited by major protein constituents such as albumin and immunoglobulin, it was sufficiently sensitive for the detection of sCD36 in whole, unfractionated and unenriched PFP.

To begin to determine the nature of sCD36 we employed size exclusion chromatography (SEC) with gel filtration of PFP on Sepharose CL-4B since this medium excludes particulate material and is a recognized method for the isolation of MPs without their possible disruption by high-speed centrifugation [26]. In this method, MPs are eluted in the void volume and soluble proteins are progressively eluted in subsequent fractions. When we subjected PFP to such SEC, subsequent protein analysis showed the void volume to contain virtually no detectable protein, with the progressively eluted fractions 17-40 containing the bulk of the protein (Fig.1A). After concentrating the void fractions by ultracentrifugation, the pellet and each of the other fractions of SEC were subjected to Western blotting using anti-CD36 antibody. CD36 could be detected only in the void volume and not in any of the fractions containing soluble proteins (Fig.1B), consistent with sCD36 in PFP being associated with circulating MPs.

CD36 functions as a receptor for various lipoproteins and long-chain fatty acids [4, 12]. A possible explanation for the exclusion of sCD36 during SEC is that it may bind to its lipid ligands to form small liposomes or remnant-like lipoprotein particles-cholesterol [27, 28] which then are eluted in the void volume [29]**.** To examine this, PFP was clarified by a short ultracentrifugation step, then the lipoproteins separated by isopycnic ultracentrifugation in an Optiprep gradient. Following fractionation, equal volumes of each sample were analysed for their lipoprotein content by agarose gel electrophoresis (AGE) and Sudan black staining (Fig.2A), and by Western blotting for CD36 (Fig.2B). As seen in Figure 2, although the different lipoprotein constituents were well separated and abundantly present, no associated sCD36 was identified even after prolonged exposure of the Western blot. This result suggests that sCD36 is not bound to its lipoprotein ligand in human plasma.

The foregoing pointed to the source of sCD36 as being MPs. Therefore, circulating MPs were isolated from PFP by ultracentrifugation and the resulting MPs pellet together with donor-matched platelets (n=6) subjected to Western blotting analysis for CD36. A strong immunoreactive band at ~88 kDa decorating CD36 was observed in both plasma MPs and platelet samples as illustrated by two representative subjects, I and II in Figure 3A. The same samples were also immunoblotted for CD41 and this platelet antigen was present in both platelet and plasma MPs fractions (Fig.3A). These experiments establish that CD36 co-precipitates with platelet MPs from the PFP of normal donors and that it migrates with the same apparent Mr as membrane-bound platelet CD36. Corroborating these results, application of PFP to a dual specificity ELISA assay demonstrated that CD41 specific signals were readily detected after capturing antigens using a CD36 mAb (Supplementary Fig.1B).

Next, we sought to identify sCD36 in the soluble fraction of plasma after separation of the MPs from soluble plasma proteins by ultracentrifugation. Whole platelet lysate was again used as a positive control and all samples analysed by Western blot for CD36. CD36 was present in the MP pellet fraction at the same Mr as platelet CD36 (Fig.3B, compare platelets and (a)). However, no sCD36 was detected in the supernatant even after using an anti-CD36 immunoprecipitation strategy to concentrate possibly low levels of sCD36 (Fig.3B(b)). Similarly, all detectable ELISA reactivity against both CD36 and CD41 was abolished when MPs were removed from PFP by ultracentrifugation (Supplementary Fig.1B). The efficiency and specificity of the 11H5 mAb used for immunoprecipitation and in the ELISA capture were demonstrated using whole PFP (Fig.3B(c) and (d)).

Taken together, the data above indicate that sCD36 is a component of MPs rather than being a product of proteolytic cleavage or a distinct isoform. Nevertheless, to exclude that either such product was somehow produced and simply associated with MPs in our fractionation procedures, we further analysed the MP-associated CD36. A cleaved CD36 product with both transmembrane domains removed would be expected to run at a lower Mr than platelet CD36 and this was not the case (see Fig.3A). Even the removal of only the C-terminal transmembrane domain and tail in an artificially expressed CD36 construct resulted in a secreted product of Mr 78 KDa as compared to the 88KDa of platelet CD36 [30]. However, since glycosylation accounts for nearly 40% of the mass of CD36 [2] and can vary with different cell types [31], this analysis may be insufficient to exclude the possibility that the cell free circulating CD36 had undergone some manner of proteolytic cleavage, yet exhibits a similar Mr because of differences in its glycosylation. Therefore, PNGase-F was used to cleave all N-linked

glycosylated residues in the samples before analysis. It was found that CD36 from both plasma MPs and platelets resolved at an identical Mr indicating that no proteolytic cleavage of the polypeptide backbone had occurred (Fig.3C).

To further supplement these data we undertook an analysis of platelet MPs and soluble proteins released after activation under experimental conditions according to the scheme outlined in Figure 4A. Washed platelets were activated with either PMA or thrombin and the low speed (3000g) pellet and supernatant fractions analysed by Western blot for both CD36 and PTA1/CD226. The latter is a transmembrane receptor expressed by platelets [32] and has been reported to be found in plasma in soluble form [33]. This analysis (Fig. 4B) showed that increased amounts of both CD36 and PTA1 could be detected in the supernatant fraction following platelet activation. While CD36 found in the supernatant was identical in size to CD36 detected in the platelet pellet, analysis of PTA1 showed that much of the PTA1 in the supernatant had greatly reduced electrophoretic mobility indicative of cleavage. Further fractionation of the low speed supernatant was then undertaken using ultracentrifugation to separate MPs from soluble proteins (Fig.4C). As expected, all of the CD36 protein released by platelets pelleted as MPs while most intact PTA1 was associated with MPs and most cleaved PTA1 remained in the supernatant fraction. Consistent with these results, analysis of microparticles by flow cytometry showed a similar increase in the release of CD36+ MPs following platelet activation (Suppl.Fig.2).

Discussion

Herein it is shown that soluble CD36, reported as a significant plasma marker for insulin resistance and diabetes [15, 16], is not truly soluble but is a constituent of circulating MPs. Many cell surface receptors that have been subjected to proteolytic cleavage and release circulate as a ligand-bound complex. For example, soluble human insulin receptor ectodomain (α subunit) which is a product of proteolytic cleavage circulates in a form bound to insulin in the plasma of diabetic subjects [34]. However, it is shown that CD36 does not associate with its fractionated lipoprotein ligands at least, therefore it appears unlikely that "soluble" CD36 is either soluble or circulating as a ligand-bound complex.

This result was not entirely unexpected. CD36 is known to be highly resistant to proteases, and was first characterized as a protease-resistant platelet surface glycoprotein. In addition, even in the absence of its two putative transmembrane domains, the molecule contains a long stretch of hydrophobic residues which has been postulated to associate with the plasma membrane [31]; such a stretch is unlikely to remain exposed on a circulating soluble protein in plasma. Further substantiating this conclusion, experiments on isolated platelets showed that CD36 was only released in the form of MPs following activation with different agonists and was not cleaved (Fig.4). In contrast, PTA1, another platelet marker reported to be found plasma [32], was substantially cleaved following platelet activation into a soluble form.

The finding that sCD36, a marker for insulin resistance and diabetes, is not in fact soluble is not an entirely negative result. Since we have demonstrated that circulating CD36 is associated with MPs, this finding will now enable more detailed analysis of the source of the

CD36+MPs. In Figure 3B we demonstrated that CD36+MPs could readily be immunoprecipitated and the presence of CD36 confirmed by immunoblotting for CD36 with a different mAb. In principle, this technique can be extrapolated to identify any other cellular antigen present on the CD36+ MPs –by first immunoprecipitating with anti-CD36, and then Western blotting the lysed precipitate with antibodies specific for different cell types. Proof of principle of this technique is shown in Figure 5A where we have immunoprecipitated the CD36+MPs from two normal subjects then blotted for CD36, followed by stripping of the filter and re-probing with anti-CD41, a platelet-specific marker. From this it is readily apparent that at least a proportion of CD36+MPs are derived from platelets. The established technique of flow cytometric analysis of MPs [24, 25] can also readily be adapted for the analysis of CD36+MPs in plasma by running directly-labeled CD36 mAb as one of the parameters, as illustrated in Figure 5B. As shown, the CD36+MPs from this normal subject are predominantly Annexin V and CD41+, but low for CD62e and CD14, indicating that most derive from activated platelets and few from activated endothelial cells or monocytes in this example. Such analyses of the plasma from subjects with insulin resistance and diabetes will prove informative. For example, Koga et al have demonstrated that platelet-derived MPs are elevated in lean Japanese patients with type 2 diabetes mellitus, and they attribute this to an increase in remnant-like lipoprotein particles-cholesterol which can activate platelets to release MPs [28]. Therefore, it may be found that the increased “soluble” CD36 identified as a plasma marker for insulin resistance and diabetes may simply reflect a methodologically different approach yielding the same conclusion. Alternatively, however, it has been shown that CD36 expressed by kidney proximal tubular epithelial cells (PTEC) is an essential mediator of the apoptosis of these cells [14]. Such apoptosis of PTEC results in tubular epithelial degeneration, a hallmark of the progression of

diabetic nephropathy, a common complication of type 1 and type 2 diabetes mellitus and the most common cause of kidney failure. Apoptosing cells release MPs [5, 6] and, since the signal for apoptosis by PTEC is ligand uptake by CD36 on the cell surface [14], it can be anticipated that any MPs released by the apoptotic PTEC will be CD36+. Therefore, by a combination of CD36 and PTCE antigen markers, simple analyses such as those in figure 4 may enable the early detection of diabetic nephropathy in patients with type 1 and type 2 diabetes.

Conclusions

This study establishes that the reported circulating "soluble" CD36 in human plasma is found in the form of circulating MPs that originate mainly from platelets in normal subjects. Increased circulating CD36 is considered to be a novel marker of insulin resistance. Therefore, we propose that such CD36 identifies a specific subset of circulating MPs. This finding will enable the establishment of the source of origin of the increased CD36+MPs associated with insulin resistance and diabetes.

Acknowledgements

This study was supported by a University of Newcastle Strategic Pilot Grant and a Hunter Medical Research Institute (HMRI) Special Project Grant - supported by Lions District 201 N3 Diabetes Foundation awarded to RFT and LFL. MJK is supported by a scholarship from the Jordan University of Science and Technology (JUST).

References

1 Okumura T, Jamieson GA. Platelet glycocalicin. I. Orientation of glycoproteins of the human platelet surface. *J Biol Chem*. 1976; **251**: 5944-9.

2 Tandon NN, Lipsky RH, Burgess WH, Jamieson GA. Isolation and characterization of platelet glycoprotein IV (CD36). *J Biol Chem*. 1989; **264**: 7570-5.

3 Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem*. 1993; **268**: 11811-6.

4 Abumrad NA, el-Maghrabi MR, Amri EZ, Lopez E, Grimaldi PA. Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. *J Biol Chem*. 1993; **268**: 17665-8.

5 Febbraio M, Silverstein RL. CD36: implications in cardiovascular disease. *Int J Biochem Cell Biol*. 2007; **39**: 2012-30.

6 Greenwalt DE, Watt KW, So OY, Jiwani N. PAS IV, an integral membrane protein of mammary epithelial cells, is related to platelet and endothelial cell CD36 (GP IV). *Biochemistry*. 1990; **29**: 7054-9.

7 Gruarin P, De Monte L, Alessio M. CD36 folding revealed by conformational epitope expression is essential for cytoadherence of Plasmodium falciparum-infected red blood cells. *Parasite Immunol*. 2000; **22**: 349-60.

8 Oquendo P, Hundt E, Lawler J, Seed B. CD36 directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes. *Cell*. 1989; **58**: 95-101.

9 Abumrad NA. CD36 may determine our desire for dietary fats. *J Clin Invest*. 2005; **115**: 2965-7.

10 Tao N, Wagner SJ, Lublin DM. CD36 is palmitoylated on both N- and C-terminal cytoplasmic tails. *J Biol Chem*. 1996; **271**: 22315-20.

11 Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med*. 2000; **6**: 41-8.

12 Calvo D, Gomez-Coronado D, Suarez Y, Lasuncion MA, Vega MA. Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL. *J Lipid Res*. 1998; **39**: 777-88.

13 Febbraio M, Hajjar DP, Silverstein RL. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *J Clin Invest*. 2001; **108**: 785-91.

14 Susztak K, Ciccone E, McCue P, Sharma K, Bottinger EP. Multiple metabolic hits converge on CD36 as novel mediator of tubular epithelial apoptosis in diabetic nephropathy. *PLoS Med*. 2005; **2**: e45.

15 Handberg A, Levin K, Hojlund K, Beck-Nielsen H. Identification of the oxidized low-density lipoprotein scavenger receptor CD36 in plasma: a novel marker of insulin resistance. *Circulation*. 2006; **114**: 1169-76.

16 Handberg A, Norberg M, Stenlund H, Hallmans G, Attermann J, Eriksson JW. Soluble CD36 (sCD36) clusters with markers of insulin resistance, and high sCD36 is associated with increased type 2 diabetes risk. *J Clin Endocrinol Metab*. 2010; **95**: 1939-46.

17 Handberg A, Skjelland M, Michelsen AE, Sagen EL, Krohg-Sorensen K, Russell D, Dahl A, Ueland T, Oie E, Aukrust P, Halvorsen B. Soluble CD36 in plasma is increased in patients with symptomatic atherosclerotic carotid plaques and is related to plaque instability. *Stroke*. 2008; **39**: 3092-5.
18 Fernandez-Real JM, Handberg A, Ortega F, Hojlund K, Vendrell J, Ricart W. Circulating soluble CD36 is a novel marker of liver injury in subjects with altered glucose tolerance. *J Nutr Biochem*. 2009; **20**: 477-84.
19 Jimenez-Dalmaroni MJ, Xiao N, Corper AL, Verdino P, Ainge GD, Larsen DS, Painter GF, Rudd PM, Dwek RA, Hoebe K, Beutler B, Wilson IA. Soluble CD36 ectodomain binds negatively charged diacylglycerol ligands and acts as a co-receptor for TLR2. *PLoS One*. 2009; **4**: e7411.
20 Wang L, Bao Y, Yang Y, Wu Y, Chen X, Si S, Hong B. Discovery of antagonists for human scavenger receptor CD36 via an ELISA-like high-throughput screening assay. *J Biomol Screen*. 2010; **15**: 239-50.
21 Thorne RF, Zhang X, Song C, Jin B, Burns GF. Novel immunoblotting monoclonal antibodies against human and rat CD36/fat used to identify an isoform of CD36 in rat muscle. *DNA Cell Biol*. 2006; **25**: 302-11.
22 Jia W, Liu XS, Zhu Y, Li Q, Han WN, Zhang Y, Zhang JS, Yang K, Zhang XH, Jin BQ. Preparation and characterization of mabs against different epitopes of CD226 (PTA1). *Hybridoma*. 2000; **19**: 489-94.
23 Thorne RF, Meldrum CJ, Harris SJ, Dorahy DJ, Shafren DR, Berndt MC, Burns GF, Gibson PG. CD36 forms covalently associated dimers and multimers in platelets and transfected COS-7 cells. *Biochem Biophys Res Commun*. 1997; **240**: 812-8.
24 Robert S, Poncelet P, Lacroix R, Arnaud L, Giraudo L, Hauchard A, Sampol J, Dignat-George F. Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies? *J Thromb Haemost*. 2009; **7**: 190-7.
25 Lacroix R PP, Robert S, Key NS, Dignat-George F. . Standardization of flow cytometry-based enumeration of platelet microparticles using calibrated beads: results of instrument qualification. *Journal of Thrombosis and Haemostasis*. 2009; **7**: Supp.2.
26 Dean WL, Lee MJ, Cummins TD, Schultz DJ, Powell DW. Proteomic and functional characterisation of platelet microparticle size classes. *Thromb Haemost*. 2009; **102**: 711-8.
27 Rigotti A, Acton SL, Krieger M. The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. *J Biol Chem*. 1995; **270**: 16221-4.
28 Koga H, Sugiyama S, Kugiyama K, Fukushima H, Watanabe K, Sakamoto T, Yoshimura M, Jinnouchi H, Ogawa H. Elevated levels of remnant lipoproteins are associated with plasma platelet microparticles in patients with type-2 diabetes mellitus without obstructive coronary artery disease. *Eur Heart J*. 2006; **27**: 817-23.
29 Scherphof G, Roerdink F, Waite M, Parks J. Disintegration of phosphatidylcholine liposomes in plasma as a result of interaction with high-density lipoproteins. *Biochim Biophys Acta*. 1978; **542**: 296-307.
30 Pearce SF, Wu J, Silverstein RL. A carboxyl terminal truncation mutant of CD36 is secreted and binds thrombospondin: evidence for a single transmembrane domain. *Blood*. 1994; **84**: 384-9.
31 Greenwalt DE, Lipsky RH, Ockenhouse CF, Ikeda H, Tandon NN, Jamieson GA. Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood*. 1992; **80**: 1105-15.

32 Scott JL, Dunn SM, Jin B, Hillam AJ, Walton S, Berndt MC, Murray AW, Krissansen GW, Burns GF. Characterization of a novel membrane glycoprotein involved in platelet activation. *J Biol Chem*. 1989; **264**: 13475-82.
33 Ye X, Zhang Z, Jiang Y, Han X, Wang Y, Zhang M, Liu J, Geng W, Dai D, Shi W, Shang H. Expression of human CD226 on T cells and natural killer cells and of soluble CD226 in plasma of HIV-1-infected Chinese patients. *Viral Immunol*. 2006; **19**: 576-81.
34 Soluble insulin receptor ectodomain is elevated in the plasma of patients with diabetes. *Diabetes*. 2007; **56**: 2028-35.

Figure Legends

Fig. 1. sCD36 in PFP is associated with circulating MPs isolated by size exclusion chromatography. (A) Protein analysis of PFP size exclusion fractions showed the void volume to contain almost no detectable protein. Fractions of the void volume were pooled and concentrated by ultracentrifugation as circulating MPs are expected to be eluted in the void volume. (B) The pellet of the void volume and each of other fractions of size exclusion were subjected to Western blotting for CD36. By this analysis CD36 was detected only in the pellet of the void volume suggesting that sCD36 is associated with circulating MPs.

Fig. 2. Isolated plasma lipoproteins show no association with sCD36. (A) Optiprep-density gradient fractions of PFP collected from normal fasting donors were analysed by AGE. Lipoproteins were revealed by Sudan Black staining. (B) Corresponding lipoprotein fractions were also analysed by Western blotting for CD36 without prior immunoprecipitation. Prolonged exposures showed no lipoprotein-associated CD36. AGE; agarose gel electrophoresis, VLDL; very low density lipoprotein, LDL; low density lipoprotein, HDL; high density lipoprotein.

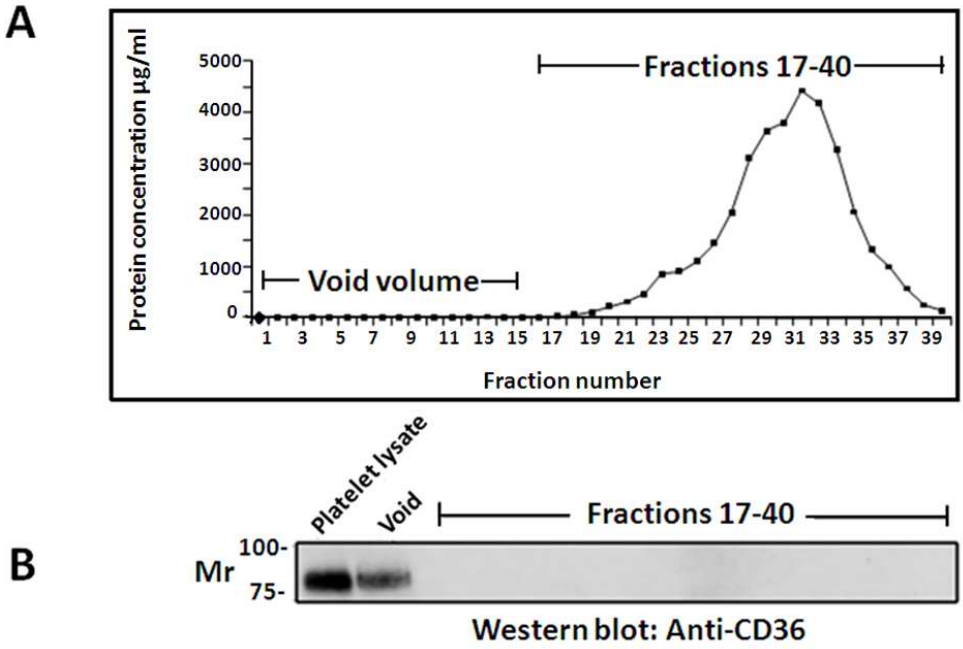
Fig. 3. sCD36 is a component of circulating MPs rather than being a proteolytic product. (A) Platelet lysate and plasma MPs were isolated from plasma of two normal donors (I & II). Samples were Western blotted for the platelet specific CD41 and CD36. Results show detectable CD41and CD36 in plasma MPs as well as platelets. This suggests CD36 co-precipitate with platelet MPs in normal donors and it has similar Mr as platelet CD36. (B) Schematic of the experimental procedure used. After removing MPs from PFP by ultracentrifugation, both the pellet and the supernatant were Western blotted for CD36. No CD36 was detected in the supernatant even after it had been enriched

by immunoprecipitation. The efficiency and specificity of the antibody used for immunoprecipitation were demonstrated using whole PFP. (C) Western blotting revealed that the CD36 molecule presents in platelets and MPs preparations was a ~88 kDa protein that is typical of membrane bound CD36. Platelets and MPs samples were mock digested or digested with PNGase-F. Pre-treatment with PNGase-F to cleave N-linked glycosylated residues resulted in similar band shifts, suggesting that the sCD36 was identical to that on the surface of platelets, and thus unlikely to be a cleaved product.

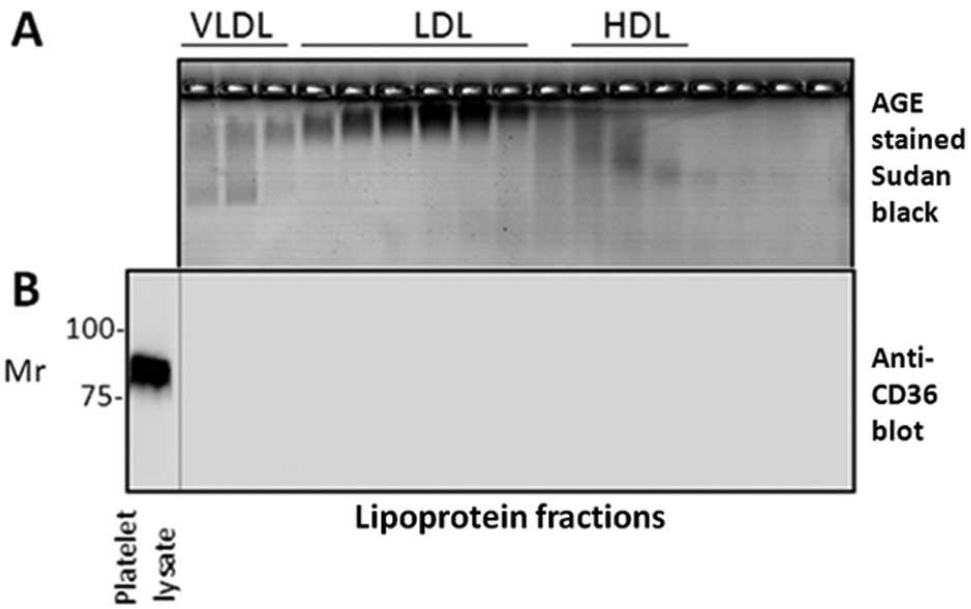
Fig.4. sCD36 released after platelet activation is associated only with MPs. (A) Schematic showing the experimental scheme after which all samples were analysed by Western blotting for CD36 and PTA1. (B) Washed human platelets treated with the indicated agonists for 10min (PMA and Thrombin) were fractionated by low speed centrifugation at 3000g to prepare pellets and supernatants. The results show that sCD36 in the supernatant fraction increased after platelet activation and was similar in Mr to CD36 detected in the platelet pellets. PTA1 levels also increased in supernatants after activation, but in contrast to CD36, PTA1 was detected in both intact and cleaved forms. (C) The low-speed supernatants from (B) were further fractionated using ultracentrifugation to isolate pellet and supernatant fractions containing MPs and soluble proteins, respectively. All sCD36 and most of the intact PTA1 released by platelets was detected with the MP pellet fraction whereas cleaved PTA1 was mainly found in the supernatant fraction.

Fig. 5. Determination of the source of CD36+MPs in normal subjects. (A) CD36+MPs were isolated by immunoprecipitation from PFP of two different donors (I & II) and Western blotted for CD36 and platelet specific CD41. CD41 was co-immunoprecipitated with CD36+MPs suggesting that at

least a proportion of the CD36+MPs are originated from platelets. (B) Representative analysis of MPs in PFP of a healthy donor labelled with CD36 antibody and other antibodies against platelets (CD41), activated endothelium (CD62e), monocytes (anti CD14), and annexin V. The figure shows that most of the CD36+MPs detected are annexin V and CD41 positive but low for CD62e and CD14, suggesting that most are derived from platelets and few from endothelial cells or monocytes. Similar results were recorded in 26 healthy subjects.



82x56mm (300 x 300 DPI)



82x52mm (300 x 300 DPI)

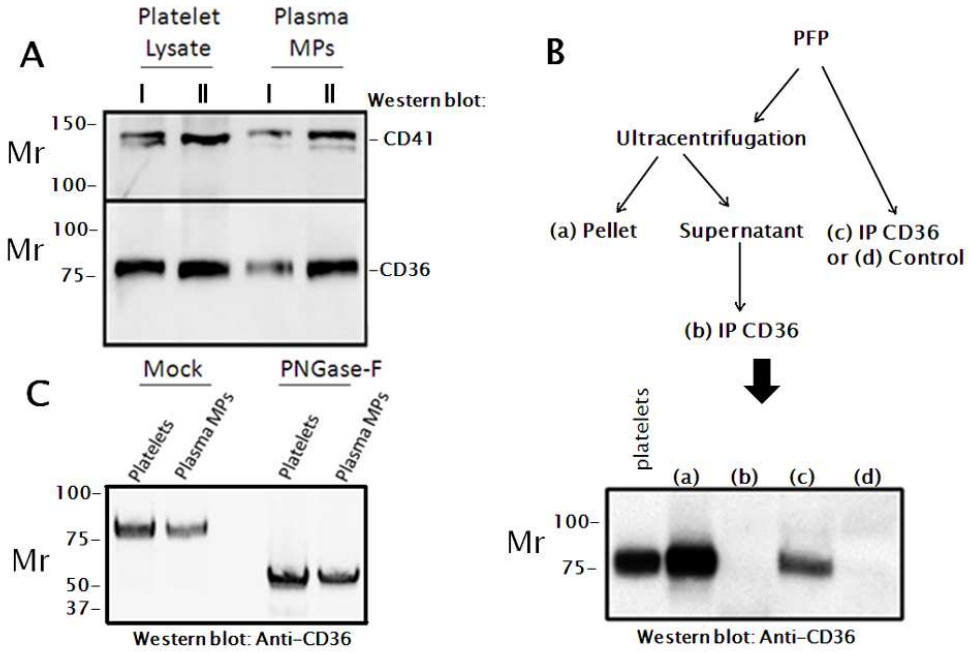


Figure 3
256x180mm (96 x 96 DPI)

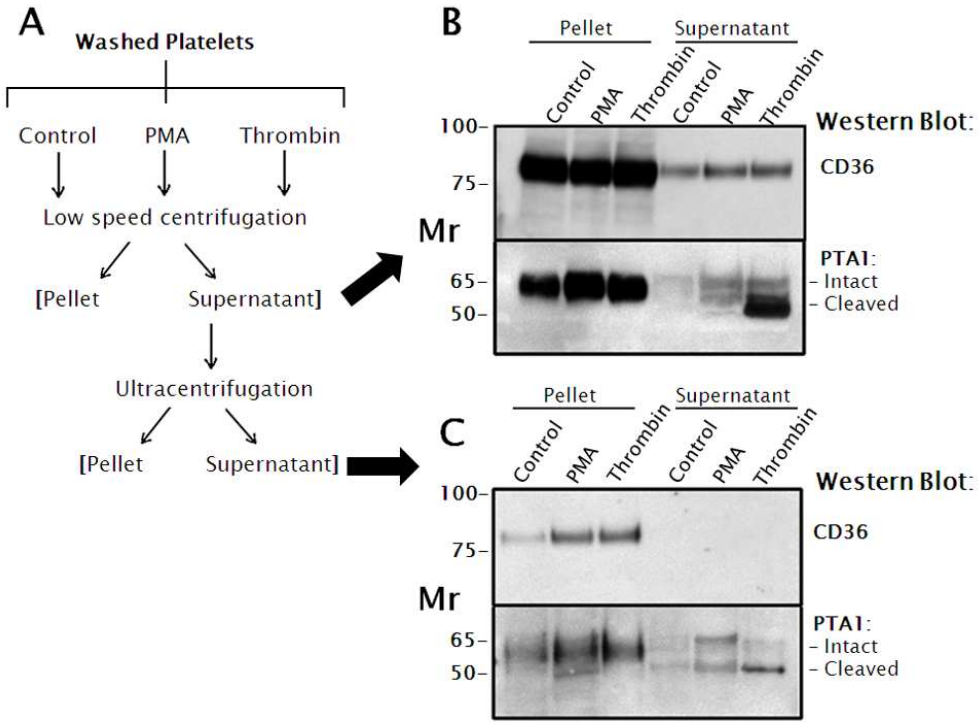


Figure 4
343x262mm (72 x 72 DPI)

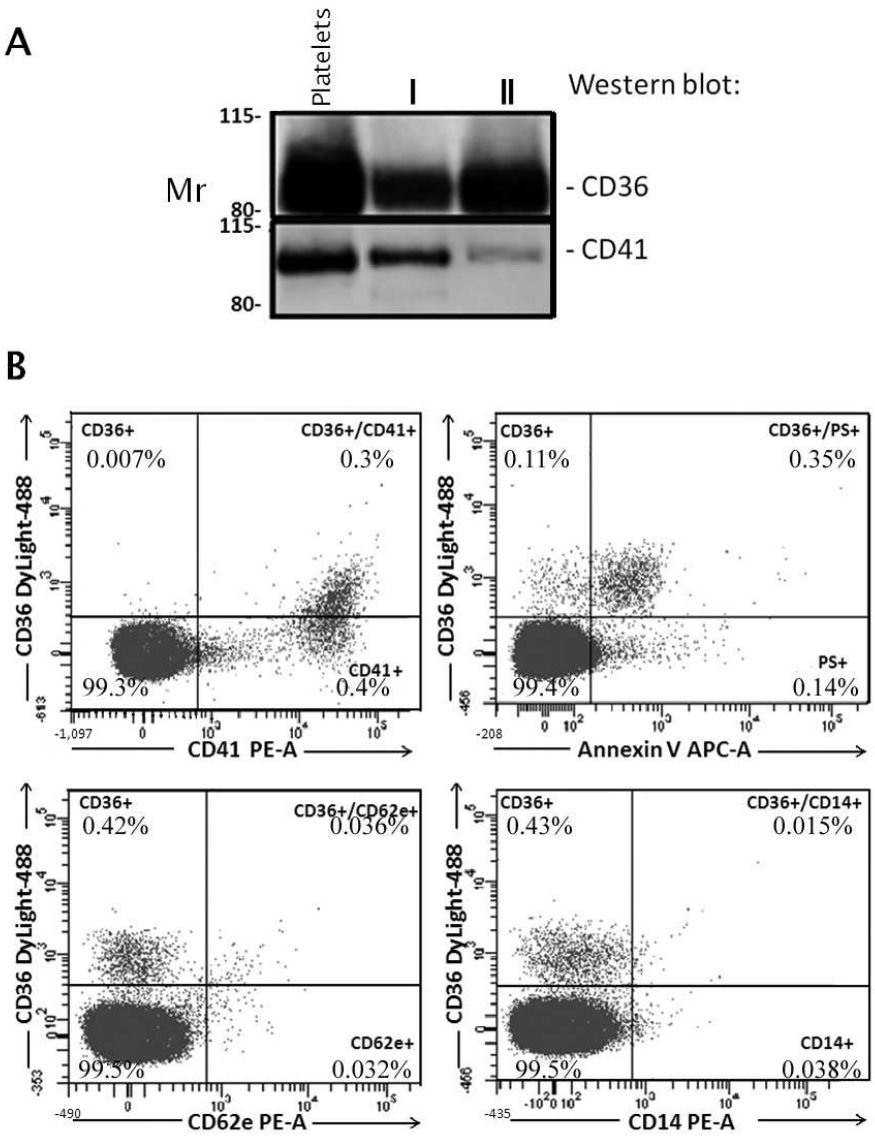
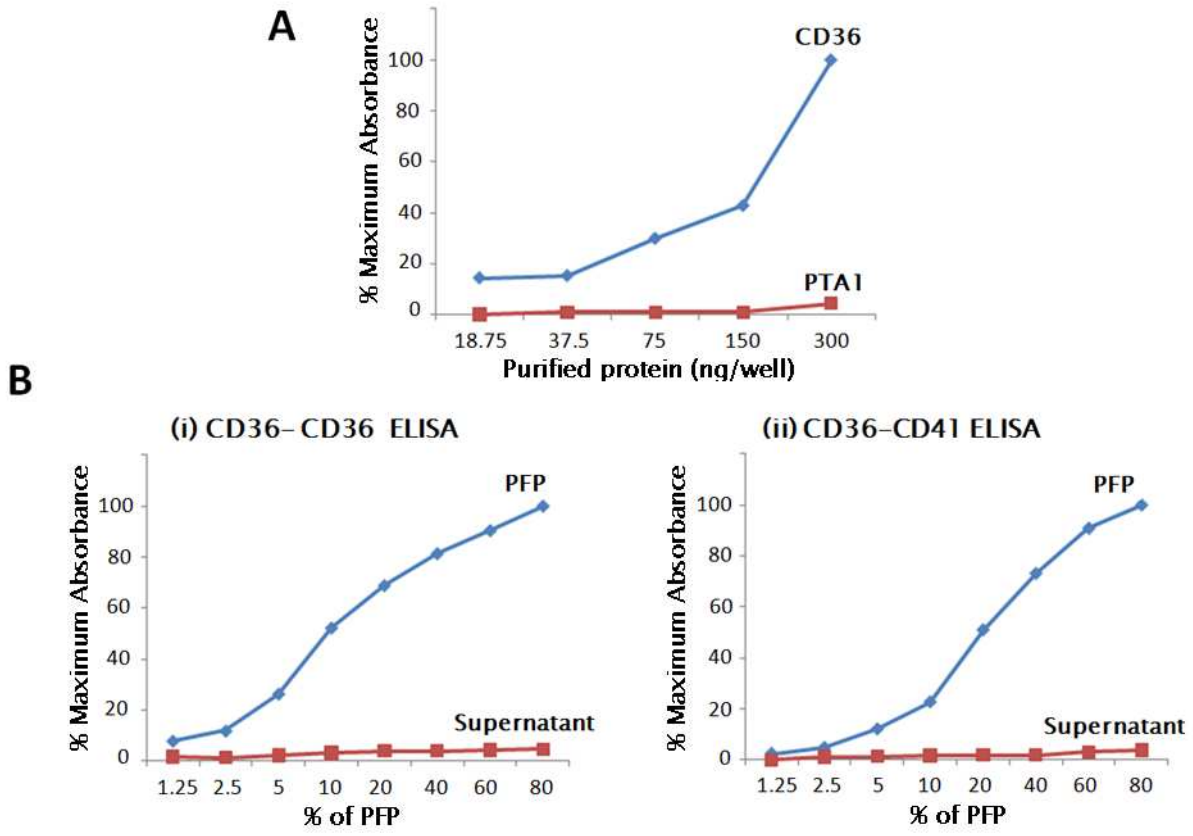
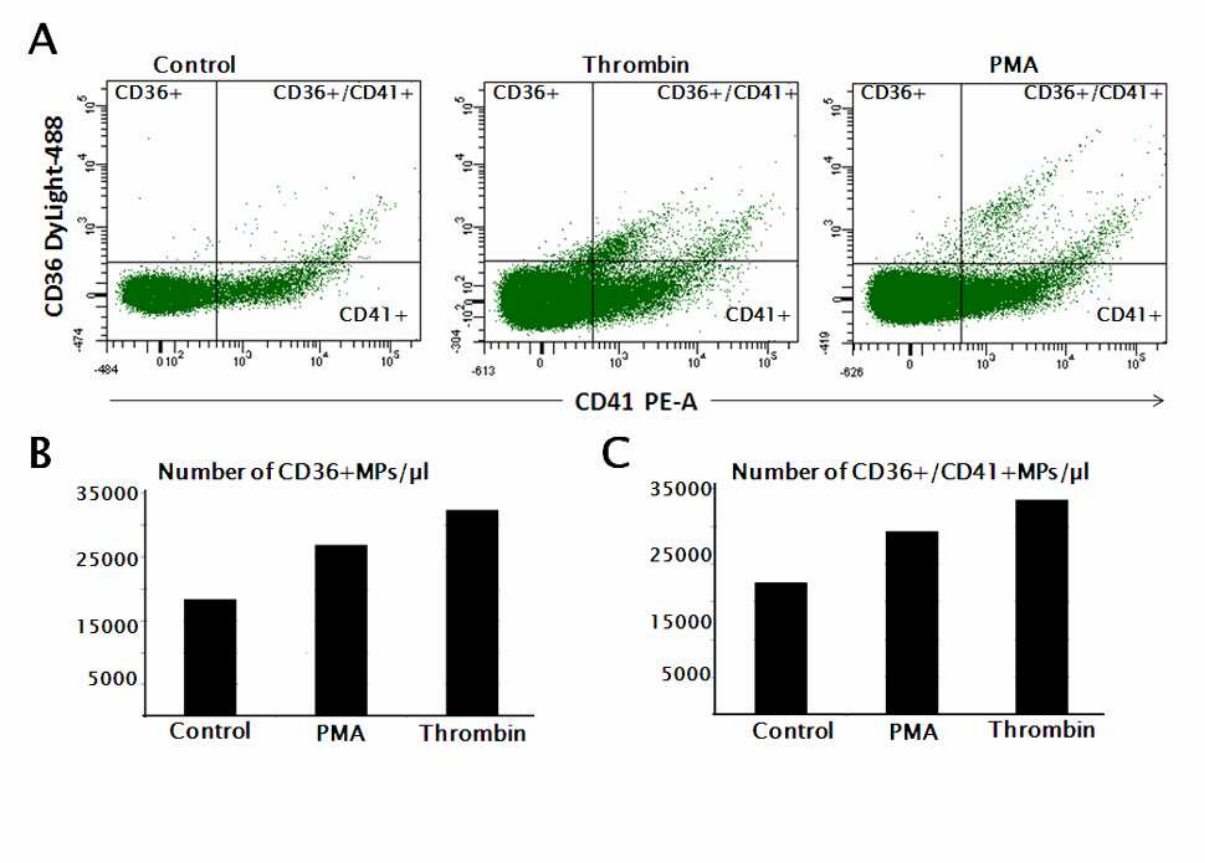


Figure 5
82x95mm (300 x 300 DPI)



Supplementary Fig.1. Detection of CD36+MPs in an ELISA assay

(A) Validation of the specificity of the ELISA assay described in the Materials and Methods. Immunoaffinity purified platelet proteins were applied to the CD36 capture - CD36 detection ELISA (CD36-CD36) assay at the indicated concentrations. Reactivity was observed against the CD36 protein but not towards the PTA1 control protein indicating that the ELISA reaction was specific for CD36 and not other antigens. (B) Dilutions of PFP and the supernatant fraction of PFP after ultracentrifugation were applied to (i) the CD36-CD36 ELISA or (ii) the CD36-CD41 ELISA. The results show that specific CD36 ELISA signals could be detected down to ~1:40-1:80 dilution of PFP. Similarly, a CD41 signal was detected from PFP applied to the CD36 capture - CD41 detection ELISA. However after ultracentrifugation to remove MPs, the Supernatant fraction of PFP demonstrated no reactivity in either assay. All sample points represent the average of duplicate determinations with the results representative of at least two independent experiments. Absorbance values have been normalised to the maximal signal detected in each assay.



Supplementary Fig.2. Activation of platelets results in increased production of CD36+MPs.

(A) Representative dual colour flow cytometric analysis of MPs using CD36 and platelet-specific CD41 antibodies following activation of platelets with PMA and thrombin. The figure shows that MPs numbers were markedly increased after activation. Quantification of total MPs as either (A) total CD36+ events or (C) CD36+/CD41+ events show both classifications of MPs are increased after platelet activation. The absolute numbers of MPs/µl of interest were calculated as the number of stained events x (beads concentration/number of beads counted) after spiking samples with a known concentration of 1026 beads/µl (Count Fluorospheres, Beckman Coulter).