

1 **Inflammatory adipocyte-derived extracellular vesicles promote leukocyte**  
2 **attachment to vascular endothelial cells**

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30

1 **Abstract**

2 **Background and Aims:** Obesity is associated with an increased risk of  
3 cardiovascular disease, but the mechanisms involved are not completely  
4 understood. In obesity, the adipocyte microenvironment is characterised by both  
5 hypoxia and inflammation. Therefore, we sought to determine whether extracellular  
6 vesicles (EVs) derived from adipocytes in this setting might be involved in mediating  
7 cardiovascular disease, specifically by promoting leukocyte attachment to vascular  
8 endothelial cells.

9 **Methods:** Mature 3T3-L1 adipocytes were incubated for 24 hours under control,  
10 TNF- $\alpha$  (30 ng/mL), hypoxia (1% O<sub>2</sub>), or TNF- $\alpha$ +hypoxia (30 ng/mL, 1% O<sub>2</sub>)  
11 conditions. EVs were isolated by differential ultracentrifugation and analysed by  
12 nanoparticle tracking analysis. Primary human umbilical vein endothelial cells  
13 (HUVECs) were treated with EVs for 6 hours before being lysed for Western blotting  
14 to investigate changes in adhesion molecule production, or for use in leukocyte  
15 attachment assays.

16 **Results:** EVs from adipocytes treated with TNF- $\alpha$  and TNF- $\alpha$ +hypoxia increased  
17 vascular cell adhesion molecule (VCAM-1) production in HUVECs compared to  
18 basal level ( $4.2 \pm 0.6$  and  $3.8 \pm 0.3$ -fold increase, respectively ( $p < 0.05$ )), an effect  
19 that was inhibited by an anti-TNF- $\alpha$  neutralising antibody. Production of other  
20 adhesion molecules (E-selectin, P-selectin, platelet endothelial cell adhesion  
21 molecule and VE-Cadherin) were unchanged. Pre-incubating HUVECs with TNF-  
22  $\alpha$ +hypoxia EVs significantly increased leukocyte attachment compared to basal level  
23 ( $3.0 \pm 0.4$ -fold increase ( $p < 0.05$ )).

24 **Conclusions:** Inflammatory adipocyte EVs induce VCAM-1 production in vascular  
25 endothelial cells, accompanied by enhanced leukocyte attachment. Preventing  
26 adipocyte derived EV-induced VCAM-1 upregulation may offer a novel therapeutic  
27 target in the prevention of obesity-driven cardiovascular disease.

28 **Word Count:** 245/250

## 1 **Introduction**

2 Obesity is a risk factor for the development of cardiovascular disease (CVD)<sup>1</sup> but the  
3 complex mechanisms linking the two together are still to be fully elucidated. Obesity  
4 combined with metabolic syndrome (an umbrella term encompassing insulin  
5 resistance, dyslipidaemia, vascular endothelial dysfunction, hypertension and a  
6 hypercoagulable state), further increases the risk of CVD<sup>2, 3</sup>. Whilst a complete  
7 understanding of the mechanisms that trigger adipose tissue to become  
8 metabolically dysregulated in obesity are not fully understood, evidence suggests  
9 that hypoxia and inflammation play a role.

10 Obese adipose tissue contains localised regions of hypoxia<sup>4-7</sup> thought to arise due to  
11 increased cell number and cell size beyond the distance oxygen can diffuse from  
12 capillaries. Free fatty acids and cytokines produced by hypoxic adipocytes attract  
13 and activate macrophages and in doing so, induce local tissue inflammation<sup>8</sup>. Under  
14 such conditions, physiological adipokine production is dysregulated. For example,  
15 release of pro-inflammatory tumour necrosis factor (TNF)- $\alpha$  is increased<sup>9</sup> whilst anti-  
16 inflammatory adiponectin is decreased<sup>10</sup>. The elevation of TNF- $\alpha$  and decrease of  
17 adiponectin in plasma are individually associated with increased leukocyte  
18 attachment to vascular endothelial cells via increased production of vascular  
19 endothelial cell adhesion molecules<sup>11, 12</sup>. Subsequent phagocytosis of oxidised low-  
20 density lipoproteins within vessel walls results in lipid-laden foam cell formation<sup>13</sup> and  
21 later, atherosclerotic plaque development.

22 Like most cells, adipocytes release extracellular vesicles (EVs)<sup>14, 15</sup>. EVs are  
23 membrane-bound structures that can convey biological information from a cell of  
24 origin to a recipient cell to achieve a target effect. Based on diameter and biogenesis  
25 they are categorised into three broad groupings: exosomes (~80-100 nm; released  
26 from multivesicular bodies upon plasma membrane fusion), microvesicles (~200-  
27 1000 nm; shed directly from the plasma membrane) and apoptotic bodies (~1-5  $\mu$ m;  
28 released as blebs during apoptosis). *In vitro* data show that adipocytes release EVs  
29 containing adipokines<sup>16-19</sup>. In obesity, adipocytes reside in a hypoxic and  
30 inflammatory environment, therefore the content and function of adipocyte-derived  
31 EVs in this environment may be different to adipocyte-derived EVs from lean adipose  
32 tissue. Adipocyte EVs circulating in obesity may contribute to vascular endothelial  
33 dysfunction which later leads to atherosclerotic plaque formation. We sought to test  
34 this hypothesis *in vitro* using a leukocyte attachment assay with vascular endothelial  
35 cells isolated from umbilical cords and EVs derived from adipocytes cultured in  
36 conditions representative of lean and obese (hypoxic and inflammatory) adipose  
37 tissue.

## 1 **Materials and Methods**

2 Ethical approval for this study was granted by the NHS Health Research Authority  
3 (ethics committee reference: 14/NW/1459), and Cardiff Metropolitan University's  
4 Research Ethics Committee.

### 5 **Adipocyte Culture and EV Isolation**

6 3T3-L1 adipocytes were cultured for 14 days as described by Connolly *et al*<sup>16</sup>. For all  
7 EV-isolation experiments, serum-containing culture medium was replaced with  
8 serum-free medium for 24 hours. During this time, adipocytes were exposed to one  
9 of four treatments representative of control (95 % air / 5 % CO<sub>2</sub>), inflamed (30 ng/mL  
10 TNF- $\alpha$  (13473019, ThermoFisher, USA)), hypoxic (1 % O<sub>2</sub>), and inflamed and  
11 hypoxic (30 ng/mL TNF- $\alpha$ , 1 % O<sub>2</sub>) adipose tissue. Cell media were centrifuged at 4  
12 °C at 1,000 *g* (5 minutes), 15,000 *g* (15 minutes), and finally 100,000 *g* (1 hour) to  
13 pellet EVs. EVs were resuspended in 1 $\times$  PBS and quantified by nanoparticle tracking  
14 analysis (NTA; a technique that tracks nanoparticles in suspension using a laser,  
15 and based on Brownian motion, determines particle size and concentration). NTA  
16 was performed using a NanoSight LM10 with a 642 nm laser (Malvern Instruments  
17 Ltd, Malvern, UK), using software version 2.3, screen gain 4 and camera level 10.  
18 Five, one-minute videos were recorded per sample with analysis screen gain 10 and  
19 detection threshold 4. Temperature ranged from 20 to 23.5 °C.

### 20 **HUVEC Isolation and Culture**

21 Human umbilical cords were obtained following elective Caesarean sections. Saline  
22 was used to flush blood out of the umbilical vein, before one end was clamped.  
23 Collagenase type 1A (1 mg/mL; C5894, Sigma) in Medium 199 (M199; 31150,  
24 ThermoFisher) at 37 °C, was syringed into the vein until the cord became taut and  
25 then the end of the cord was also clamped. After 15 minutes, one clamp was  
26 released allowing the vascular endothelial cell suspension to be collected into a  
27 Falcon tube. Copious growth medium (M199 containing 10 % (v/v) foetal bovine  
28 serum (10500-064, ThermoFisher), human epidermal growth factor (1 ng/mL;  
29 13453029, ThermoFisher), hydrocortisone (1  $\mu$ g/mL; H0888, Sigma), gentamycin (35  
30  $\mu$ g/mL; G1272, Sigma) and amphotericin (0.5  $\mu$ g/mL; A2942, Sigma)) was added to  
31 terminate the enzymatic digestion. Cells were centrifuged (300 *g*, 5 minutes, 4 °C),  
32 resuspended in growth medium, and plated in 96-well plates for leukocyte  
33 attachment assays or 6-well plates for protein assays. Plates were pre-coated with 1  
34 % (w/v) bovine skin gelatin (G9391, Sigma) in 1 $\times$  PBS. HUVECs were given 2 hours  
35 to adhere, before the medium was aspirated and replaced. HUVECs reached  
36 confluency within 4-7 days, were never passaged, and were used for experiments  
37 within 7 days.

### 38 **Leukocyte Isolation**

39 Blood (10 mL) was obtained from healthy volunteers by venepuncture and  
40 transferred into a universal container (UC) containing Heparin (100  $\mu$ L, 5,000 I.U./mL,

1 Wockhardt, India). Dextran (2.5 mL, 6 % (w/v), Sigma) dissolved in 1× balanced salt  
2 solution (BSS; 0.13 M NaCl, 2.6 mM KCl, 8.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.83 mM KH<sub>2</sub>PO<sub>4</sub>, pH  
3 7.4) was added and mixed by a single inversion. Blood was transferred into a fresh  
4 UC and allowed to fractionate for 1 hour. The buffy coat layer (~1.5 mL) was  
5 transferred into a fresh UC. Cells were collected by centrifugation (300 g, 2 minutes,  
6 room temperature) and the pellet resuspended in sterile H<sub>2</sub>O to burst any  
7 contaminating erythrocyte membranes. After 10 seconds the UC was filled with BSS  
8 and the leukocytes were pelleted as above. The pellet was resuspended in Krebs-  
9 BSA (0.1 % (w/v) bovine serum albumin (BSA) in 1× Krebs (1.2 M NaCl, 0.48 M KCl,  
10 0.12 M KH<sub>2</sub>PO<sub>4</sub>, 0.12 M MgSO<sub>4</sub>, 0.13 M CaCl<sub>2</sub>, 2.5 M HEPES, pH 7.4)) that had  
11 been passed through a 0.22 µm pore sterile-filter (Merck Millipore, USA) for  
12 sterilisation and remove possible contaminating serum extracellular vesicles. Cells  
13 were incubated on ice, in darkness, with CellTrace™ (1:1000; C34851, Invitrogen).  
14 After 10 minutes, leukocytes were centrifuged, resuspended in Krebs-BSA and left to  
15 settle on ice for 30 minutes. Prior to the attachment assay, the leukocyte suspension  
16 was diluted 1:10 with Krebs-BSA pre-warmed to 37 °C.

### 17 **Leukocyte Attachment Assay**

18 Serum-free M199 (150 µL) containing  $1.2 \pm 0.4 \times 10^{10}$  (Control),  $2.5 \pm 0.4 \times 10^{10}$   
19 (TNF-α),  $8.6 \pm 0.8 \times 10^{10}$  (Hypoxia) and  $1.2 \pm 0.3 \times 10^{11}$  (TNF-α & Hypoxia) adipocyte  
20 EV/mL (mean ± SEM; N=3, n=9), was added to individual wells of a 96-well plate  
21 containing a confluent monolayer of HUVECs. Wells were also used to determine  
22 basal leukocyte attachment (no EVs; negative control) or 100 ng/mL TNF-α to  
23 increase HUVEC VCAM-1 production (no EVs; positive control). After 6 hours, EVs  
24 were removed with three Krebs washes before 150 µL of the fluorescently-labelled  
25 leukocyte suspension was added to all wells. Plates were incubated for 30 minutes  
26 before non-adherent cells were removed with three Krebs washes. Three images  
27 from around the centre of each well were captured using an inverted fluorescence  
28 microscope. The percentage of total image area covered by leukocytes was  
29 determined using Image J software (1.49v; National Institutes of Health, USA).  
30 Incubation timings were based on published protocols<sup>20</sup>. Three separate sets of  
31 adipocyte-derived EVs were used in this experiment and each set of EVs was tested  
32 on three HUVEC samples (N=3, n=9).

### 33 **Effect of Adipocyte EVs on HUVEC Adhesion Molecule Production**

34 Serum-free M199 (1.5 mL) containing  $1.2 \pm 0.4 \times 10^{10}$  (Control),  $2.5 \pm 0.4 \times 10^{10}$   
35 (TNF-α),  $8.6 \pm 0.8 \times 10^{10}$  (Hypoxia) and  $1.2 \pm 0.3 \times 10^{11}$  (TNF-α & Hypoxia) adipocyte  
36 EV/mL (mean ± SEM, N=3, n=3), was added to individual wells of a 6-well plate of  
37 HUVECs for 6 hours. Another well was used for the determination of basal protein  
38 production (no EVs; negative control). EVs were removed with three 1× PBS washes  
39 before HUVECs were lysed using radioimmunoprecipitation assay buffer (Invitrogen)  
40 containing protease inhibitors (Roche, Switzerland). Lysates were centrifuged  
41 (12,000 g, 10 minutes, 4 °C) and supernatants frozen until required. The protein  
42 concentration of each sample was determined using a NanoDrop spectrophotometer

1 and samples analysed by Western blotting. Lysates were mixed with sample buffer  
2 and reducing agent (Invitrogen) and heated (95 °C, 10 minutes). Ten µg of total  
3 protein from each sample was loaded onto 4-12 % Bis-Tris gels (Novex™,  
4 ThermoFisher) and SDS-PAGE performed (180 V, 1 hour). Resolved proteins were  
5 transferred to polyvinylidene difluoride membranes (40 V, 75 minutes). Membranes  
6 were blocked using 5 % (w/v) non-fat dried milk in 1× Tris-buffered saline containing  
7 0.05 % Tween-20 (TBST) (1 hour). Primary antibodies directed at proteins of interest  
8 (vascular cell adhesion molecule (VCAM-1; ab134047), E-selectin (ab18981), P-  
9 selectin (ab59738), platelet endothelial cell adhesion molecule (PECAM; ab28364),  
10 vascular endothelial (VE)-cadherin (ab33168), endothelial nitric oxide synthase  
11 (eNOS; ab76198) (all Abcam, Cambridge, UK)) were diluted 1:500 in blocking buffer  
12 and incubated with membranes overnight. Membranes were then incubated with  
13 horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1000; 10794347,  
14 ThermoFisher) (2 hours). Protein bands developed on enhanced chemiluminescence  
15 film (10607665, Fisher Scientific). Membranes were re-probed for β-actin (1:2000;  
16 4970S, Cell Signalling Technology (CST), Netherlands) to confirm equal loading.  
17 Band densitometry was performed using Image J. Three sets of adipocyte-derived  
18 EVs were used in this experiment with each set tested on one HUVEC sample (N=3,  
19 n=3).

## 20 **Effect of Adipocyte EVs on the Plasma Membrane Expression of HUVEC** 21 **Adhesion Molecules**

22 HUVECs were treated with 3T3-L1 EVs from each condition (control, TNF-α,  
23 hypoxia, TNF-α+hypoxia) as described above, then washed twice and detached from  
24 the culture plate using 200 µL 1× PBS. HUVECs were collected by centrifugation  
25 (300 g, 5 minutes) and then re-suspended in ice-cold FACS Buffer (1% BSA (v/v) in  
26 1× PBS). Flow cytometry was used to assess the surface adhesion molecule profile  
27 of HUVECs. Antibodies used for flow cytometric analysis were obtained from  
28 Biolegend® (BioLegend, San Diego, CA, USA). They include; phycoerythrin (PE)  
29 anti-human CD144, allophycocyanin (APC) anti-human VCAM-1, Alexafluor 647 anti-  
30 human PECAM-1, PE anti human E-Selectin, and PECy7 anti-human ICAM-1. Cells  
31 were incubated with antibodies for 30 minutes at 4 °C in darkness before being  
32 analysed on an FC500 MPL flow cytometer (Beckman Coulter equipped with 488 nm  
33 and 633 nm lasers) and data captured on MPX Cytometer List Mode Data  
34 Acquisition and Analysis Software (version 2.2). Acquisition was terminated upon  
35 recording 10,000 events, and cells gated based on their forward scatter and side  
36 scatter characteristics. Fluorescence minus one (FMO) stains were used to set the  
37 positive gates for each antibody (See supplemental information and supplementary  
38 Figure 3 for gating strategy). Three separate sets of adipocyte- derived EVs were  
39 used in this experiment and each set of EVs was tested on one individual HUVEC  
40 sample (N=3, n=3). Mean Fluorescence Intensity (MFI) was used to compare  
41 surface marker expression between treatments.

42

## 1 **TNF- $\alpha$ Neutralisation Assay**

2 A TNF- $\alpha$  neutralising antibody was used to determine whether EVs derived from  
3 adipocytes treated with TNF- $\alpha$ , mediated VCAM-1 upregulation in HUVECs. To  
4 neutralise any pre-existing TNF- $\alpha$ , HUVECs and adipocyte-derived EVs were  
5 incubated separately in serum-free culture medium containing 0, 3, 10, 30, 100 or  
6 300 ng/mL TNF- $\alpha$  neutralising antibody. After 2 hours, HUVEC media were removed  
7 and the EV-containing media were added to the corresponding wells. After 6 hours,  
8 HUVECs were lysed and VCAM-1 levels assessed by Western blotting, as  
9 previously described. Four sets of adipocyte-derived EVs were used in this  
10 experiment with each set tested on one HUVEC sample (N=4, n=4).

## 11 **Analysis of 3T3-L1 Cell and EV Proteins**

12 Adipocyte cell and EV lysates were assessed by Western blotting as previously  
13 described. Ten  $\mu$ g of total protein was loaded per lane for cell lysates, and 5  $\mu$ g of  
14 total protein for EV lysates. Primary antibodies directed at fatty acid binding protein-4  
15 (FABP4; 3544S, CST), adiponectin (2789S, CST), peroxisome proliferator-activated  
16 receptor gamma (PPAR $\gamma$ ; 2443S, CST) and perilipin (9349S, Cell Signalling  
17 Technology), were used at a 1:500 dilution, and HRP-conjugated anti-rabbit IgG was  
18 used at a 1:1000 dilution. Three sets of adipocyte cell and EV lysates were used  
19 (N=3, n=3).

## 20 **Statistical Analyses**

21 Data are presented as mean or mode  $\pm$  SEM. A one-way ANOVA with Tucky's  
22 Multiple Comparison Test was used to analyse differences. Data were analysed  
23 using GraphPad Prism (version 6; GraphPad Software Inc., USA) and *p*-values  
24 <0.05 were considered significant.<sup>20</sup>

## 1 **Results**

### 2 **Effect of Adipocyte EVs on Leukocyte-to-Endothelial Cell Attachment**

3 HUVECs pre-treated with TNF $\alpha$ +hypoxia-derived adipocyte EVs, increased  
4 leukocyte attachment to the same extent as the positive control (Figure 1A). These  
5 increases were significant when compared to those observed for HUVECs not  
6 treated with EVs and HUVECs pre-treated with control adipocyte EVs (Figure 1A).  
7 Leukocyte attachment was also greater following treatment with TNF $\alpha$ +hypoxia-  
8 derived EVs compared to hypoxia-derived adipocyte EVs. Pre-treating HUVECs with  
9 TNF- $\alpha$ -derived adipocyte EVs also increased leukocyte attachment compared to  
10 HUVECs not treated with EVs. No other differences were observed in leukocyte  
11 attachment to HUVECs after pre-treatment with adipocyte EVs. Using size-exclusion  
12 chromatography, we confirm that TNF- $\alpha$  is associated with EVs; there is no free  
13 TNF- $\alpha$  (Supplementary Figure 1).

### 14 **Effect of Adipocyte EVs on HUVEC Protein Production**

15 Following treatment of HUVECs with adipocyte EVs, Western blotting was used to  
16 examine the production of proteins involved in leukocyte attachment (Figure 2).  
17 VCAM-1 production was increased in HUVECs treated with TNF- $\alpha$  EVs and TNF-  
18  $\alpha$ +hypoxia EVs compared to that of untreated HUVECs. No change was observed  
19 with control EVs nor hypoxia EVs (Figure 2A). The production of other adhesion  
20 proteins including E-selectin (Figure 2B), P-selectin (Figure 2C) and PECAM (Figure  
21 2D) were unaffected by treatment with adipocyte EVs. In addition, no effect on the  
22 production levels of the vascular endothelial cell marker proteins VE-Cadherin  
23 (Figure 2E) and eNOS (Figure 2F) were identified.

### 24 **Effect of Adipocyte EVs on the Plasma Membrane Expression of HUVEC** 25 **Adhesion Molecules**

26 Flow cytometry confirmed the expression of VCAM-1, E-selectin, PECAM-1 and  
27 ICAM-1 on HUVEC plasma membranes (Figure 3). However, there were no  
28 statistically significant differences in the mean fluorescence intensity for each protein  
29 between any of the treatment groups. TNF- $\alpha$  treatment (no EVs) of HUVECs was  
30 used as a positive control in all experiments and showed positive expression of  
31 adhesion markers, other than for PECAM-1 which showed high expression at  
32 baseline and following all treatment conditions.

### 33 **TNF- $\alpha$ Neutralisation Prevents VCAM-1 Upregulation in HUVEC**

34 To determine if the upregulation of VCAM-1 in HUVECs seen following treatment  
35 with TNF- $\alpha$  and TNF- $\alpha$ +hypoxia adipocyte EVs (Figure 2) is due to EV-associated  
36 TNF- $\alpha$ , a TNF- $\alpha$  neutralising antibody was used. Compared to untreated HUVECs,  
37 TNF- $\alpha$ +hypoxia EVs increased VCAM-1 production (Figure 4). Compared to the level  
38 of VCAM-1 production achieved following treatment with TNF- $\alpha$ +hypoxia EVs, co-  
39 treatment with 100 ng/mL and 300 ng/mL neutralising antibody completely inhibited  
40 VCAM-1 upregulation (Figure 4).



1 **Adipokine Production in Adipocytes and Adipocyte-Derived EVs**

2 No effect on the production of FABP4 in cell lysates due to any treatment was  
3 identified (Figure 5A). FABP4 in was increased in EVs lysates of cells treated with  
4 TNF- $\alpha$  regardless of normoxia or hypoxia (Figure 5B). Adiponectin production was  
5 decreased in cells treated with TNF- $\alpha$ +hypoxia compared to that of control cells, but  
6 individually, TNF- $\alpha$  and hypoxia had no effect (Figure 5C). All treatments appeared  
7 to decrease adiponectin in EV lysates compared to that of control cells (Figure 5D).  
8 PPAR $\gamma$  production in cell lysates decreased in response to hypoxia and TNF-  
9  $\alpha$ +hypoxia (Figure 5E). Conversely, hypoxia- and TNF- $\alpha$ +hypoxia-derived EVs  
10 contained more PPAR $\gamma$  (Figure 5F). Compared to that of control cells, perilipin  
11 production decreased in response to hypoxia and TNF- $\alpha$ +hypoxia (Figure 5G). No  
12 differences in the production of perilipin in EVs were identified (Figure 5H).

13 **Effect of Inflammatory and Hypoxic Stimuli on Adipocyte EV Yield and Size.**

14 Compared to control adipocytes, treatment with TNF- $\alpha$ , hypoxia and TNF- $\alpha$ +hypoxia  
15 all increased the number of EVs produced per cell (Figure 6A). Treatment with TNF-  
16  $\alpha$ , hypoxia, or TNF- $\alpha$ +hypoxia all decreased EV size compared to control adipocytes  
17 (Figure 6B).

## 1 **Discussion**

2 Increased adiposity has long been recognised as a risk factor for cardiovascular  
3 disease development<sup>1</sup>. In obesity, adipose tissue inflammation is associated with  
4 vascular inflammation<sup>21</sup> and one of the primary mediators of this process is the  
5 inflammatory cytokine, TNF- $\alpha$ . Whilst TNF- $\alpha$  is secreted by adipocytes themselves,  
6 levels in obese adipose tissue are predominantly raised by activated macrophages<sup>22</sup>.  
7 TNF- $\alpha$  desensitizes adipocytes to insulin<sup>23</sup>, but distally and in terms of effects on  
8 vascular endothelial cells, plasma TNF- $\alpha$  levels positively correlate with adhesion  
9 molecule production, disrupted eNOS activity and oxidative stress<sup>24</sup>. We are the first  
10 to show that EVs derived from adipocytes residing within a hypoxic and  
11 inflammatory, TNF- $\alpha$ -containing environment (modelling the pathophysiological  
12 inflammatory nature of obese adipose tissue *in vivo*), are implicated in the onset of  
13 vascular disease by increasing vascular endothelial cell adhesion molecule  
14 production and thereby promoting leukocyte attachment. In addition, we show that  
15 inflammatory and hypoxic stimuli affect the content of adipocyte-derived EVs, as well  
16 as their yield and size.

17 Leukocyte-to-endothelial cell attachment was increased following treatment of  
18 HUVECs with TNF- $\alpha$  and TNF- $\alpha$ +hypoxia derived adipocyte EVs when compared to  
19 untreated HUVECs. However, when compared to the level of leukocyte attachment  
20 following treatment of HUVECs with control EVs, attachment was only increased  
21 following treatment with TNF- $\alpha$ +hypoxia EVs. This suggests that a factor(s)  
22 conferred by hypoxia, can elicit a functional effect on vascular endothelial cells that  
23 exacerbates leukocyte adhesion in the presence of inflammation, and this is worthy  
24 of future investigation. The data do however, indicate that inflammatory EVs,  
25 regardless of whether they were produced under normoxic or hypoxic conditions,  
26 prime endothelial cells for subsequent leukocyte attachment.

27 Whilst flow cytometry confirmed the expression of the adhesion molecules VCAM-1,  
28 E-selectin, PECAM-1 and ICAM-1 on the surface of HUVEC plasma membranes, no  
29 differences between EV treatment groups were observed. Although not significant  
30 VCAM-1 surface expression did, however, show a trend to increase in cells treated  
31 with TNF- $\alpha$  and TNF- $\alpha$ +hypoxia derived adipocyte EVs and mirrored the increase in  
32 VCAM-1 detected by Western blotting. We hypothesize that in order to detect subtle  
33 changes in surface marker expression between treatment groups will likely require a  
34 very high number of HUVEC/experimental replicates and it is acknowledged further  
35 studies will be required in order to investigate differences in surface expression.

36 The fact that inflammatory adipocyte EVs increase HUVEC VCAM-1 production,  
37 does however, offer a mechanism through which leukocyte-to-endothelial cell  
38 attachment is achieved. TNFR1 receptors have been shown to induce VCAM-1  
39 mRNA and protein via a pathway mediated by NF- $\kappa$ B but not ERK, p38MAPK or JNK  
40 kinase<sup>25</sup>. Future experiments aim to determine if the TNF- $\alpha$  delivered to HUVECs by

1 3T3-L1 EVs activates TNFR1 receptors and mediates VCAM-1 upregulation via a  
2 similar mechanism. As TNF- $\alpha$  neutralisation prevents VCAM-1 upregulation, we  
3 hypothesise that this would also prevent the increase in leukocyte-to-endothelial cell  
4 attachment following incubation of HUVEC with TNF- $\alpha$  and TNF- $\alpha$ +hypoxia derived  
5 adipocyte EVs. *In vivo*, VCAM-1 is involved in the firm attachment of leukocytes to  
6 endothelial cells prior to their transmigration through the vessel wall<sup>26, 27</sup>, and whilst  
7 enhanced VCAM-1 production accompanied by leukocyte attachment is known to  
8 contribute to the progression of atherosclerosis<sup>28, 29</sup>, we can now suggest that this is  
9 at least partly mediated by EVs derived from hypoxic and inflamed adipocytes.

10 In this study, EVs were not processed further to remove possible contaminating  
11 “free” TNF- $\alpha$ . However, we do provide evidence that TNF- $\alpha$  co-elutes with CD63  
12 and FABP-4 following size exclusion chromatography of EV samples and that no  
13 “free” TNF- $\alpha$  elutes in later fractions. This indicates that TNF- $\alpha$  within EV samples is  
14 EV-associated (Supplementary Figure 1). In addition, it is important to note that the  
15 leukocytes for the leukocyte-to-endothelial attachment assay were isolated from  
16 heparin-anticoagulated blood. Heparin may activate platelets<sup>30</sup> causing them to  
17 expose P-selectin. P-selectin can consequentially bind to P-selectin glycoprotein on  
18 leukocytes, and this may have affected leukocyte attachment<sup>31</sup>. However, this effect  
19 would be equal across all experimental conditions, and as such, the results observed  
20 in this study can be attributed to 3T3-L1 EVs. It is also important to note that once  
21 isolated from blood, the leukocytes were resuspended in 0.22  $\mu$ m sterile-filtered  
22 Krebs-BSA. As such, there is a chance that bovine serum EVs smaller than 0.22  $\mu$ m  
23 were present in this buffer and that they too could have affected leukocyte adhesion.  
24 Likewise, this effect would have been equal across all conditions and  
25 consequentially the levels of leukocyte attachment observed across all experimental  
26 conditions would remain in proportion to each other.

27 Our results add to the growing body of literature confirming that adipocytes not only  
28 release EVs<sup>15, 16, 32</sup>, but that the information conveyed through their content has a  
29 functional effect on other cell types. As such, we investigated the effect of  
30 inflammatory and hypoxic stimuli on the production of several proteins (FABP4,  
31 adiponectin, PPAR $\gamma$  and perilipin) linked to cardiovascular disease in both adipocyte  
32 cell and EV lysates. We hypothesise that differences in the content of these proteins  
33 within EVs derived from inflammatory and/or hypoxic adipocytes may confer  
34 mechanistic influences on leukocyte attachment to vascular endothelial cells. Future  
35 studies will seek to identify and elucidate such mechanisms.

36 Plasma levels of FABP4, a fatty-acid chaperone protein<sup>33</sup>, have been shown to  
37 increase in obesity<sup>34</sup> and are associated with vascular endothelial cell dysfunction<sup>35</sup>.  
38 We observed no change in the FABP4 content of adipocyte cell lysates in response  
39 to inflammatory or hypoxic stimuli. This result supports a previous study whereby  
40 FABP4 production in adipocyte cell lysates was also shown not to change in  
41 response to hypoxia<sup>36</sup>. What is interesting, however, is that the authors of this study

1 did identify an increased level of FABP4 in adipocyte culture media in response to  
2 hypoxia. In our study, hypoxia did not increase the FABP4 content of EVs suggesting  
3 that perhaps FABP4 is released by adipocytes in an EV-independent mechanism in  
4 this setting. However, we did observe an increase in FABP4 in the lysates of EVs  
5 derived from TNF- $\alpha$  treated adipocytes (regardless of normoxia or hypoxia). As such,  
6 future experiments will aim to elucidate the influence of FABP4 within inflamed  
7 adipocyte-derived EVs on vascular endothelial dysfunction.

8 Adiponectin is abundant within the circulation of healthy people and exerts regulatory  
9 insulin-sensitising and anti-inflammatory effects<sup>37,38</sup>. Plasma adiponectin levels  
10 negatively correlate with degree of obesity<sup>39</sup> and therefore also correlate with  
11 increased obesity-related co-morbidities including insulin resistance, type II diabetes  
12 and cardiovascular disease<sup>40</sup>. We found that a combined inflammatory and hypoxic  
13 stimulus decreased adiponectin in adipocyte cell lysates. EV-associated adiponectin  
14 also appeared to decrease compared to control in response to all treatments, but  
15 particularly hypoxia. Using the same adipocyte cell model, Chen *et al.*, showed that  
16 hypoxia prevents soluble adiponectin secretion<sup>40</sup>, complementing our finding. Our  
17 data add to this by suggesting that at least a proportion of this reduction in  
18 adiponectin secretion under hypoxic conditions is because it is not being packaged  
19 into EVs. Reduced adiponectin within adipocyte EVs may contribute to the decrease  
20 in plasma adiponectin during obesity and thereby may contribute to the development  
21 of cardiovascular disease.

22 PPAR $\gamma$  is a nuclear receptor regulating the transcription of genes central to fatty acid  
23 and energy metabolism<sup>41</sup>. We found that hypoxia decreased PPAR $\gamma$  production in  
24 adipocyte cell lysates. Down-regulation of intracellular PPAR $\gamma$  in response to hypoxia  
25 is well documented<sup>16, 42</sup> and is mediated by inhibition of its transcription by hypoxia  
26 inducible factor-1 (HIF-1 $\alpha$ ). Interestingly, in our study we also observed a  
27 simultaneous increase of PPAR $\gamma$  within the lysates of EVs derived from hypoxic  
28 adipocytes, confirming that adipocyte EVs are selectively packaged depending on  
29 environmental cues. We hypothesize that HIF-1 $\alpha$  may also be implicated in the  
30 selective packaging of PPAR $\gamma$  into EVs destined for release, as a further means to  
31 reduce intracellular PPAR $\gamma$  levels. Overall, this finding highlights that EVs do not  
32 necessarily possess the same content as their cell-of-origin, but rather that their  
33 content reflects the cell-of-origin's current state.

34 Perilipin is a protein localised to the surface of lipid droplets and is involved in  
35 lipolysis<sup>43</sup>. In our study, hypoxia decreased perilipin production within adipocyte cell  
36 lysates when normalised to total protein. Wang *et al.*,<sup>44</sup> previously showed no  
37 difference in total perilipin production per individual adipocyte sampled from lean and  
38 obese adipose tissue but when normalised to total protein or fat cell surface area,  
39 perilipin levels were also significantly lower in obese samples. We propose that  
40 hypoxia restricts perilipin production in adipose tissue in obesity *in vivo*. In addition,  
41 whilst perilipin levels are increased in the circulation in obese mice and humans<sup>15</sup>

1 this may be because it is released in to the circulation from adipocytes as a free  
2 protein rather than being associated with EVs. This hypothesis fits with our results as  
3 we identified no difference in the perilipin content of adipocyte EVs in response to  
4 inflammatory and hypoxic stimuli.

5 In terms of specific EV character, our group has previously shown that EVs isolated  
6 from adipocytes express the vesicular proteins CD9, CD63, Alix, tumour  
7 susceptibility gene (TSG101)<sup>16</sup>, and now show that they also exhibit characteristic  
8 cup-shape morphology by electron microscopy (Supplementary Figure 1). Here we  
9 show the yield and size of adipocyte-derived EVs can be modulated independently  
10 by external stimuli. TNF- $\alpha$ , hypoxia and TNF- $\alpha$ +hypoxia increased the yield of  
11 EVs/cell whilst decreasing their size. These physical changes may themselves also  
12 confer distinct functional effects. However, it is important to note that the size data  
13 presented is based solely on raw NTA values; no refractive index<sup>45</sup> nor mathematical  
14 modelling<sup>46</sup> was applied.

15 In summary, we provide evidence that adipocytes residing in a hypoxic and  
16 inflammatory environment produce EVs capable of inducing VCAM-1 production in  
17 vascular endothelial cells, and that this effect promotes leukocyte attachment. We  
18 also show that both inflammatory and hypoxic stimuli not only influence the  
19 adipokine content of adipocytes and their EVs, but also effect EV yield and size.  
20 Future studies will further explore the functional impact inflammatory and hypoxic  
21 adipocyte EVs have on cardiovascular disease. Indeed, the functional effects of  
22 adipocyte EVs in the setting of obesity, are likely to be not solely limited to vascular  
23 endothelial cells. We anticipate that a better understanding of the mechanisms  
24 through which adipocyte EVs negatively impact the vascular endothelium will open  
25 the door for the development of novel therapies for preventing and treating obesity-  
26 driven cardiovascular disease.

1 **Conflict of Interest**

2 The authors declare no conflict of interest.

3

4 **Financial Support**

5 This work was supported by the British Heart Foundation (grant reference:  
6 PG/14/51/30686).

7

8 **Author contributions**

9 Rebecca M. Wadey performed the experiments. Katherine D. Connolly performed  
10 pilot experiments and manuscript editing. Donna Mathew and Gareth Walters  
11 performed the flow cytometry experiments. D. Aled Rees and Philip E. James were  
12 the Principal Investigators leading the project.

13

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16 the collection of umbilical cords, the volunteers who donated blood samples, and Dr  
17 Justyna Wiczak and Mrs Margaret Munnery for performing phlebotomy. We also  
18 thank Dr Errin Johnson (EM facility, Sir William Dunn School of Pathology, University  
19 of Oxford) for performing the transmission electron microscopy.

20

21 **Figure Legends**

22 **Figure 1 – Leukocyte attachment to HUVECs pre-treated with and without**  
23 **adipocyte-derived EVs.** (A) Ratio of percentage area covered by leukocytes to a  
24 confluent monolayer of HUVECs pre-treated with (C) control, (T) TNF- $\alpha$ , (H) hypoxia,  
25 and (TH) TNF- $\alpha$ +hypoxia-derived adipocyte EVs relative to that of (B; Basal)  
26 HUVECs not treated with EVs (negative control). Direct treatment with TNF- $\alpha$  acted  
27 as a positive control (mean  $\pm$  SEM; N=3, n=9; \*\*\*  $p$ <0.001). (B) Representative  
28 fluorescent images used to determine the percentage area of HUVECs covered by  
29 leukocytes. White dots are leukocytes stained with CellTrace<sup>TM</sup>. Scale bar = 200  $\mu$ m.  
30

31 **Figure 2 – Expression of adhesion and endothelial marker proteins in HUVECs**  
32 **following treatment with adipocyte EVs.** Densitometry graphs and representative  
33 Western blots showing the ratio of (A) VCAM-1 (110 kDa), (B) E-Selectin (80 kDa),  
34 (C) P-Selectin (140 kDa), (D) PECAM (130 kDa), (E) VE-Cadherin (115 kDa) and (F)  
35 eNOS (140 kDa) expression in HUVECs following treatment with (C) control, (T)  
36 TNF- $\alpha$ , (H) hypoxia and (TH) TNF- $\alpha$ +hypoxia derived adipocyte EVs, relative to that  
37 of (B; basal) untreated HUVECs (mean  $\pm$  SEM; N=3, n=3; \*\*\*  $p$ <0.001).  $\beta$ -actin  
38 confirms equal loading (bottom blots). Ten  $\mu$ g total protein per lane.

1

2 **Figure 3 - Surface expression of HUVEC adhesion proteins following treatment**  
3 **with adipocyte EVs.** Mean fluorescence intensity graphs showing the ratio of (A)  
4 VCAM-1 (-APC), (B) E-Selectin (-PE), (C) PECAM-1 (-AlexoFluor) and (D) ICAM-1 (-  
5 PE/Cy7) expressed on the plasma membrane of HUVECs following treatment with  
6 (C) control, (T) TNF- $\alpha$ , (H) hypoxia and (TH) TNF- $\alpha$ +hypoxia derived adipocyte EVs,  
7 relative to that of (B; basal) untreated HUVECs. Direct treatment with TNF- $\alpha$  was  
8 used as a positive control (mean  $\pm$  SEM; N=3, n=3 for all except ICAM-1 where N=2,  
9 n=2).

10

11 **Figure 4 – TNF- $\alpha$  neutralisation inhibits endothelial VCAM upregulation**  
12 **following treatment with TNF- $\alpha$ +hypoxia adipocyte EVs.** (A) Western blotting  
13 densitometry of VCAM-1 expression in control HUVEC lysates, and lysates of  
14 HUVECs treated with TNF- $\alpha$ +hypoxia EVs (denoted by +) in the presence of 0, 3, 10,  
15 30, 100 and 300 ng/ml TNF- $\alpha$  neutralising antibody (mean  $\pm$  SEM; N = 4, n = 4; \*\*  
16  $p < 0.01$ ). (B) Representative VCAM-1 (110 kDa; top) and  $\beta$ -actin (40 kDa; bottom)  
17 blots. Twenty  $\mu$ g total protein per lane.

18

19 **Figure 5. FABP4, adiponectin, PPAR $\gamma$  and perilipin expression in adipocyte and**  
20 **adipocyte-derived EV lysates.** Densitometry graphs and representative Western  
21 blots for FABP4 and adiponectin in cell and EV lysates following (C) control, (T) TNF-  
22  $\alpha$ , (H) hypoxia and (TH) TNF- $\alpha$ +hypoxia treatment of adipocytes. FABP4 expression  
23 in (A) cell lysates (mean  $\pm$  SEM; N=6, n=6) and (B) EV lysates (mean  $\pm$  SEM; N=4,  
24 n=4) (15 kDa). Adiponectin expression in (C) cell lysates (mean  $\pm$  SEM; N=5, n=5; \*  
25  $p < 0.05$ ) and (D) EV lysates (mean  $\pm$  SEM; N=2, n=2) (30 kDa). PPAR $\gamma$  expression in  
26 (E) cell lysates (mean  $\pm$  SEM; N=6, n=6; \*\*\*  $p < 0.001$ ) and (F) EV lysates (mean  $\pm$   
27 SEM; N=3, n=3; \*\*  $p < 0.01$ ) (53 and 57 kDa). Perilipin expression in (G) cell lysates  
28 (mean  $\pm$  SEM; N=5, n=5; \*\*\*  $p < 0.001$ ) and (H) EV lysates (mean  $\pm$  SEM; N=3, n=3)  
29 (56 kDa). Twenty  $\mu$ g total protein per lane of cell lysates, and 5  $\mu$ g total protein per  
30 lane of EV lysates.

31

32 **Figure 6. Effect of inflammatory and hypoxic stimuli on adipocyte EV yield and**  
33 **size.** The effect of (C) control, (T) TNF- $\alpha$ , (H) hypoxia and (TH) TNF- $\alpha$ +hypoxia  
34 treatments on (A) EV yield per cell (mean  $\pm$  SEM; n=3, n=9; \*\*\*  $p < 0.001$ ) and (B) EV  
35 size (mode  $\pm$  SEM; n=3, n=9; \*\*\*  $p < 0.001$ ).

36

37

38

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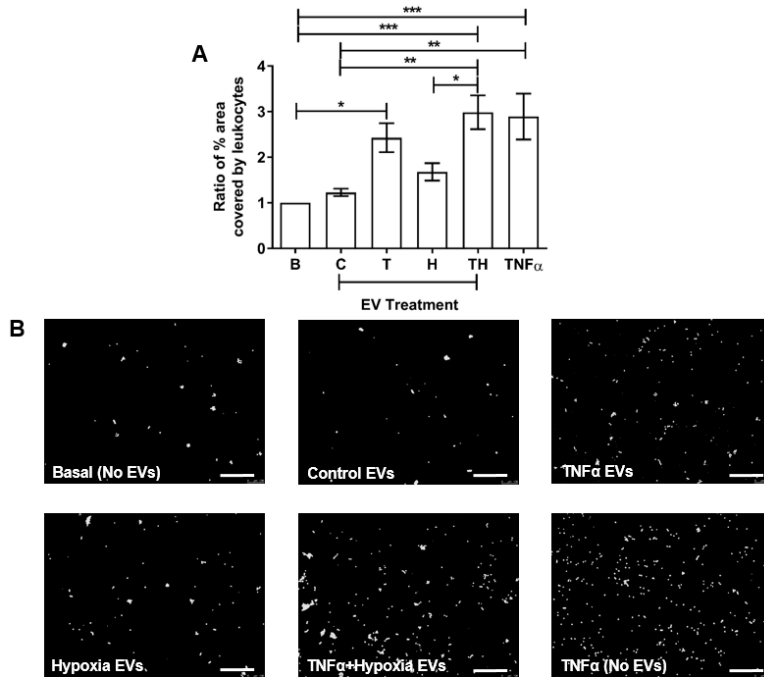
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1 **Key Words**

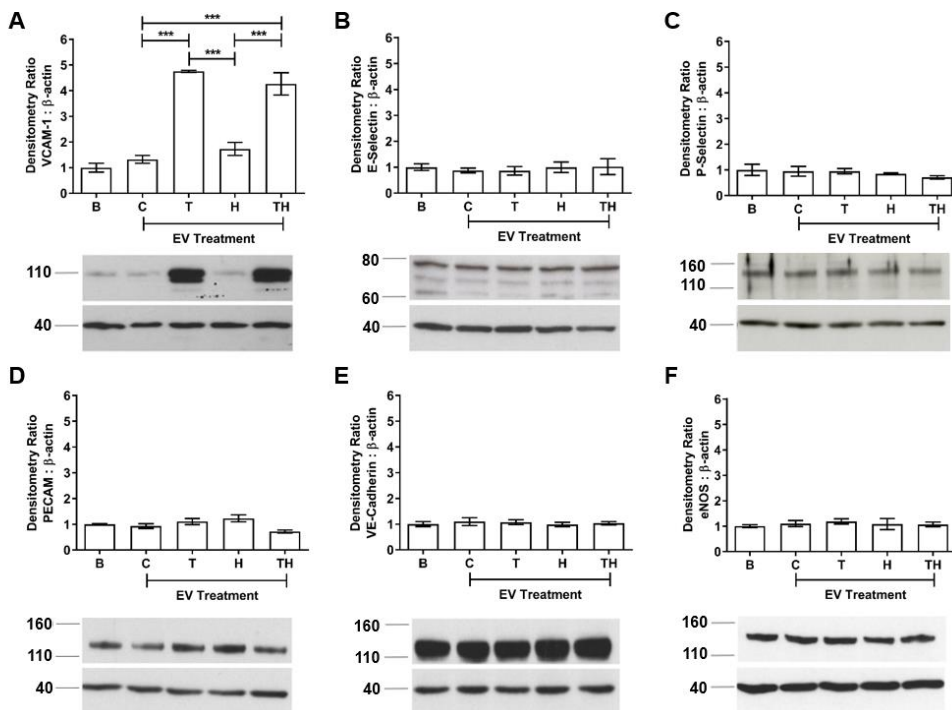
2 Adipocyte, adipokine, obesity, endothelial dysfunction, vesicle



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4 **Figure 1 – Leukocyte attachment to HUVECs pre-treated with and without adipocyte-**  
 5 **derived EVs.**

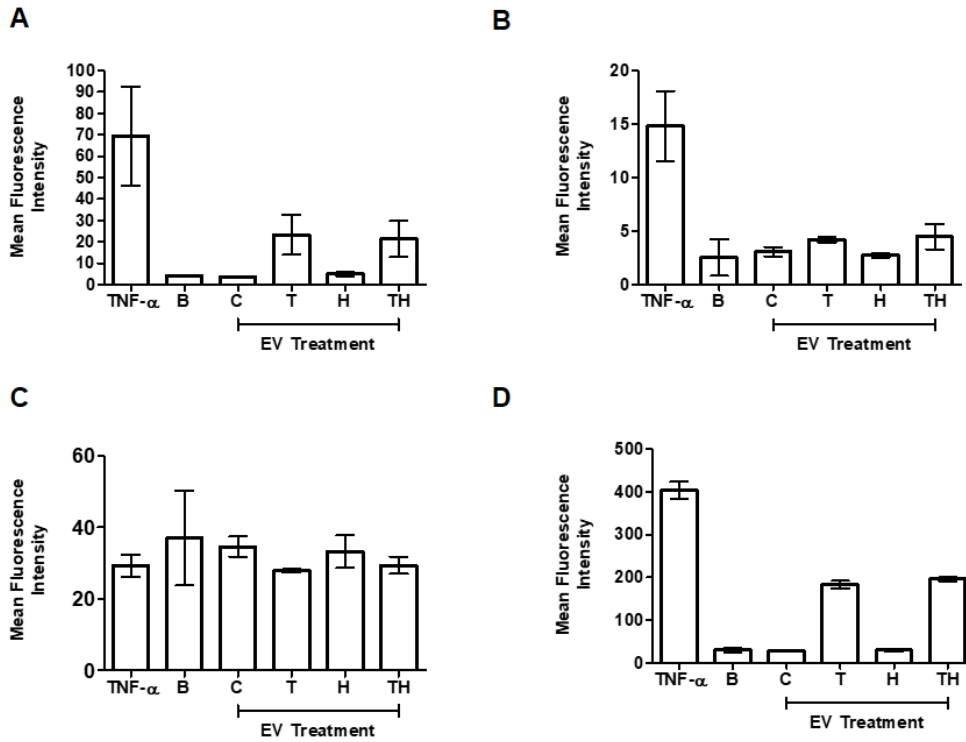
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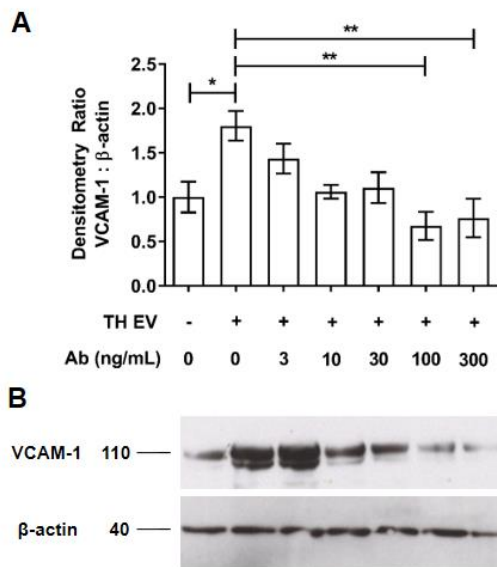
8 **Figure 2 – Expression of adhesion and endothelial marker proteins in HUVECs**  
 9 **following treatment with adipocyte EVs.**

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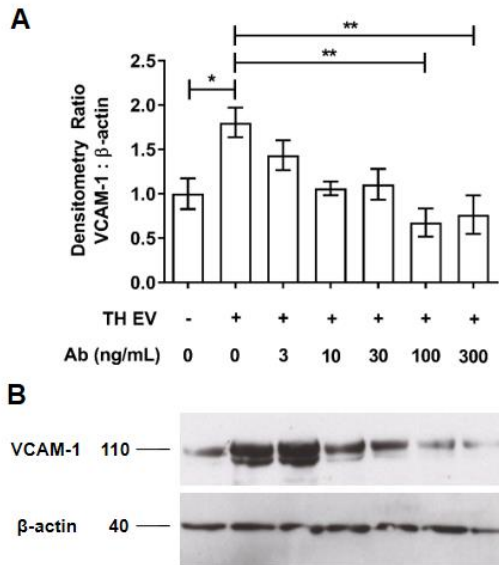
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**Figure 3 - Surface expression of HUVEC adhesion proteins following treatment with adipocyte EVs.**



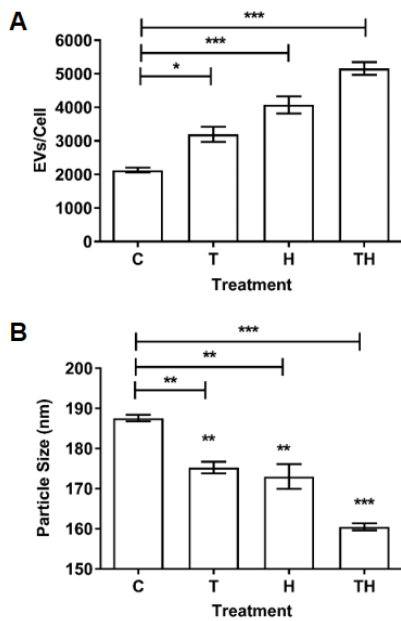
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**Figure 4 – TNF-α neutralisation inhibits endothelial VCAM upregulation following treatment with TNF-α+hypoxia adipocyte EVs.**



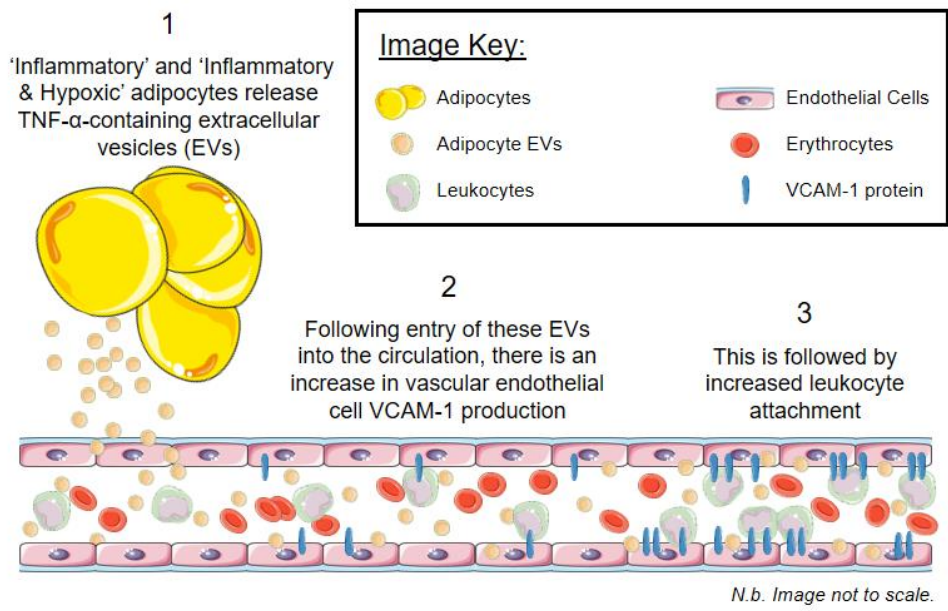
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**Figure 5. FABP4, adiponectin, PPAR $\gamma$  and perilipin expression in adipocyte and adipocyte-derived EV lysates.**



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**Figure 6. Effect of inflammatory and hypoxic stimuli on adipocyte EV yield and size.**



1

2 **Graphical Abstract**