

1 **EVIDENCE FOR ADIPOCYTE-DERIVED EXTRACELLULAR**
2 **VESICLES IN THE HUMAN CIRCULATION**

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20

21 **Abstract**

22 Adipocyte-derived extracellular vesicles (EVs) may serve as novel endocrine mediators of adipose
23 tissue and impact upon vascular health. However, it is unclear whether adipocyte-derived EVs are
24 present in the human circulation. Therefore, the purpose of this study was to seek evidence for the
25 presence of adipocyte-derived EVs in circulating plasma. Size exclusion chromatography of platelet-
26 free plasma identified fractions 5-10 as containing EVs by a peak in particle concentration, which
27 corresponded with the presence of EV and adipocyte proteins. Pooling fractions 5-10 and subjecting
28 to ultracentrifugation yielded a plasma EV sample, as verified by transmission electron microscopy
29 (TEM) showing EV structures and Western blotting for EV (e.g. CD9 and Alix) and adipocyte
30 markers. Magnetic beads and a solid phase assay were used to deplete the EV sample of the four
31 major families of circulating EVs: platelet-, leukocyte-, endothelial- and erythrocyte-derived EVs.
32 Post-depletion samples from both techniques contained EV structures as visualized by TEM, as well
33 as CD9, Alix and classic adipocyte proteins. Post-depletion samples also contained a range of other
34 adipocyte proteins from an adipokine array. Adipocyte proteins and adipokines are expressed in
35 optimally processed plasma EV samples, suggesting that adipocyte-derived EVs are secreted into the
36 human circulation.

37

38 **Précis**

39 Optimally isolated, human plasma-derived extracellular vesicles were found to contain multiple
40 adipocyte markers, even after the depletion of major circulating extracellular vesicle populations.

41

42 **Introduction**

43 The endocrine functions of adipose tissue have largely been attributed to adipokines; an array of
44 soluble bioactive molecules secreted from adipocytes such as adiponectin and fatty acid binding
45 protein (FABP)-4¹. Dysregulation of adipokine secretion is associated with obesity-related
46 cardiovascular disease, insulin resistance, and type 2 diabetes². Adiponectin, peroxisome proliferator-
47 activated receptor (PPAR)- γ , FABP4 and Perilipin have been detected within adipocyte-derived
48 extracellular vesicles (EVs) *in vitro*³⁻¹² indicating an additional method for endocrine signalling from
49 adipose tissue. Dysfunctional adipocytes in obese adipose tissue may release an altered complement
50 of EVs, which in addition to dysregulated adipokine secretion, help to promote the cardiovascular
51 complications associated with obesity. Therefore, there is a need for comprehensive evidence for the
52 existence of adipocyte-derived EVs *in vivo* to explore their potential as novel circulating biomarkers
53 of adipocytes *in vivo*.

54 EVs are heterogeneous submicron vesicles released from almost all cells in response to cellular stress,
55 activation or apoptosis. EVs may originate from cytoplasmic multivesicular bodies which fuse with
56 the plasma membrane to release vesicles typically <120 nm in diameter, often referred to as
57 exosomes. EVs also include microvesicles, which are ~ 100-1000 nm in size and bud directly from
58 the plasma membrane into the extracellular space. Both subclasses of EVs have a biomolecular
59 composition similar to that of the original cell including specific lipids, proteins, and nucleic acids.
60 Recent advances in methodology have enabled standardisation of nomenclature and characterisation
61 of EV populations¹³.

62 Most studies examining the release of EVs from adipocytes have been conducted *in vitro* using 3T3-
63 L1 cells^{3,6-9,11,12}; a murine adipocyte cell line frequently used to model adipocyte functions. Others
64 have also isolated EVs from human adipocytes and adipose tissue extracts^{4,5,10}. These studies have
65 demonstrated the functional relevance of adipocyte-derived EVs in the paracrine regulation of
66 adipocyte metabolism¹⁴, monocyte to macrophage differentiation⁴ and regulation of hepatic insulin
67 signaling⁵. Effects on vascular homeostasis have also been shown, including induction of

68 neovascularization and angiogenesis^{15,16}, suggesting that adipocyte-derived EVs may influence
69 vascular health within, and at sites remote to adipose tissue. However, evidence for the presence of
70 adipocyte-derived EVs in the human circulation has not yet been confirmed, since EVs in blood are
71 thought to derive primarily from platelets (with leukocyte-, endothelial- and erythrocyte-derived EVs
72 contributing smaller populations¹⁷⁻¹⁹), and adipocytes lack a unique marker to readily distinguish them
73 from other cells. Preliminary evidence from flow cytometric analyses showed that EVs contain the
74 adipocyte markers FABP4 and adiponectin in human and mouse plasma^{18,20}. However, the use of
75 direct flow cytometry for EV measurements is sub-optimal as the lower limit of detection for many
76 conventional flow cytometers is ~300 nm²¹, resulting in an incomplete assessment of the EV
77 population. Separate studies have also shown that adiponectin, FABP4, Perilipin and PPAR- γ were
78 associated with plasma EVs^{4,11,22} though in most cases, plasma samples were not processed in
79 accordance with guidelines set out by the International Society for Extracellular Vesicles (ISEV)¹³.
80 This may lead to false positive results from contamination of soluble adipokines present in the larger
81 plasma protein pool.

82 In light of these uncertainties, we utilized a combination of adipocyte markers and sample processing
83 according to ISEV recommendations to seek evidence for the presence of adipocyte-derived EVs in
84 healthy human plasma.

85

86 **Materials and Methods**

87 *Plasma EV isolation*

88 Ethical approval for this study was granted by Cardiff Metropolitan University School Research
89 Ethics Committee and informed consent was obtained from each volunteer. Blood was drawn from
90 seven healthy volunteers (3 males, 4 females) using a 19 G needle into 3.2% (w/v) sodium citrate
91 vacutainers and immediately centrifuged (2500 x g, 15 minutes, 21°C) to isolate platelet-poor plasma
92 (PPP). The first 3 mL of blood was discarded in line with recommended guidelines for collection of
93 EVs from blood^{23,24}. PPP was then pooled and centrifuged as above to isolate platelet-free plasma
94 (PFP). PFP (1 mL) was then loaded onto Exo-spin™ midi size exclusion columns (Cell Guidance
95 Systems, UK) and 30 x 500 µL fractions were collected. Fractions 5-10 were then pooled and
96 ultracentrifuged (100,000 x g, 1 hour, 4°C) to pellet EVs (hereafter referred to as “pooled EVs”).

97

98 *Nanoparticle Tracking Analysis*

99 Quantification of EV populations was performed using nanoparticle tracking analysis (NTA) with a
100 NanoSight LM10 instrument configured with a 488 nm laser and a sCMOS camera (Malvern
101 Instruments Ltd, UK). A Harvard Apparatus syringe pump was utilized for EV measurements at a
102 constant flow rate of 20 a.u. Camera shutter speed and gain were maintained at 607 and 15
103 respectively. Sample videos were recorded for 60 seconds in repetitions of 5 using a capture screen
104 gain of 8-11 and a camera level of 8-10. Samples were processed using a screen gain of 20 and a
105 detection threshold of 4-6. Software version 3.1 (build 3.1.54) was used for capture and analysis. All
106 experiments were performed in a temperature-controlled room at 22°C. Results are presented as
107 particles/mL.

108

109 *Western Blotting*

110 The protein concentration of individual column fractions (1-30) was determined using a NanoDrop
111 1000 Spectrophotometer (ThermoFisher Scientific, UK). Samples (8 µg) of fractions 2-28, pooled
112 EVs and post-depletion samples were prepared to 30 µL (neat or diluted with 1X PBS), boiled for 8
113 minutes on a heat block, centrifuged (12,000 x g, 5 mins, 4°C) and kept on ice before loading onto 4-
114 12% Bis-Tris gels (ThermoFisher Scientific). InstantBlue™ Protein Stain (Expedeon Ltd, UK) was
115 used as a loading control. Amersham Hybond P 0.45 µm PVDF membranes (GE Healthcare, UK)
116 were probed with the following antibodies (diluted 1:500 in either 5% (w/v) skimmed milk or 5%
117 (w/v) BSA both in tris-buffered saline with 0.05% (v/v) Tween 20): mouse monoclonal anti-Alix²⁵,
118 rabbit polyclonal anti-CD63²⁶ (both purchased from Santa Cruz Biotechnology, USA); mouse
119 monoclonal anti-CD81²⁷ (purchased from Bio-Rad, UK); rabbit monoclonal anti-Adiponectin²⁸
120 (purchased from Abcam, UK); rabbit monoclonal anti-CD9²⁹, rabbit monoclonal anti-FABP4³⁰; rabbit
121 monoclonal anti-Perilipin³¹ and rabbit monoclonal anti-PPARγ³² (purchased from Cell Signaling
122 Technologies, USA). Proteins were analysed using reducing conditions with the exception of the
123 tetraspanins (CD9, CD63 and CD81), which were analysed using non-reducing conditions. Signals
124 were detected using either goat anti-mouse IgG-HRP³³ or donkey anti-rabbit IgG-HRP³⁴ diluted
125 1:1000 in 5% (w/v) skimmed milk in tris-buffered saline with 0.05% (v/v) Tween 20) followed by
126 Amersham ECL Western Blotting Detection Reagents (GE Healthcare).

127

128 *Transmission Electron Microscopy*

129 Pooled EVs were resuspended in 1X 0.22 µm-filtered PBS and then fixed with an equal volume of 4%
130 (v/v) paraformaldehyde and kept at 4°C until processing for TEM the next day. Briefly, EVs (10 µL)
131 were adsorbed onto glow discharged carbon formvar 200 mesh copper grids for 2 minutes. Grids were
132 then blotted using filter paper, stained for 10 seconds with 2% (w/v) uranyl acetate before surplus
133 stain was removed and grids were air-dried. Grids were imaged using a FEI Tecnai 12 TEM at 120 kV
134 fitted with a Gatan OneView CMOS camera.

135 *Sequential depletion of EV populations using magnetic beads*

136 Pooled EVs were diluted to a concentration of 1×10^{11} particles/mL using 1X 0.22 μm -filtered PBS in
137 replicates of three. EVs were then incubated for 2 hours at room temperature with 3 $\mu\text{g}/\text{mL}$ rabbit
138 monoclonal anti-CD41 antibody³⁵ (purchased from Abcam). Fifty μL (per sample) of pre-washed
139 Dynabeads™ M-280 sheep anti-rabbit IgG magnetic beads (Life Technologies, UK) were added to
140 EVs/anti-CD41 and incubated with mixing for 30 minutes at room temperature. Samples were then
141 introduced into the magnet (DynaMag™-2, Life Technologies) to deplete CD41+ EVs: this was
142 quantified using NTA. The process was repeated sequentially with 3 $\mu\text{g}/\text{mL}$ rabbit monoclonal anti-
143 CD11b³⁶, rabbit polyclonal anti-CD144³⁷ and rabbit monoclonal anti-CD235a³⁸ antibodies (all
144 purchased from Abcam) to deplete CD11b+, CD144+ and CD235a+ EVs. Final supernatants were
145 quantified using NTA and analysed by Western Blot with “pre-depletion” samples for the presence of
146 adipocyte and EV markers.

147

148 *Solid-phase-based depletion of EV populations*

149 High binding ELISA plates (Greiner Bio-One Ltd, UK) were coated in triplicate with rabbit
150 monoclonal anti-CD41, -CD11b, -CD144 or -CD235a antibodies (Abcam, as above) diluted to 3
151 $\mu\text{g}/\text{mL}$ in PBS overnight at 4°C. Pooled EVs were diluted to a concentration of 1×10^{11} particles/mL
152 as above and incubated for 2 hours at room temperature in wells containing anti-CD41 antibody to
153 deplete CD41+ EVs. Supernatants were then transferred to wells containing anti-CD11b antibody for
154 2 hours at room temperature to deplete CD11b+ EVs. This process was then repeated sequentially
155 with wells containing anti-CD144 antibody and anti-CD235a antibody to deplete CD144+ and
156 CD235a+ EVs. Final supernatants were analysed as above.

157

158 *Time Resolved Fluorescence (TRF)*

159 The efficiency of depletion of major circulating EV populations was assessed using time resolved
160 fluorescence (TRF) as previously described^{39,40}. Briefly, EVs were normalised to a concentration of
161 1×10^{11} particles/mL in pre-depletion, post-CD41, post-CD11b, post-CD144 and post-CD235a samples
162 from magnetic bead and solid phase-based depletion. EVs were then immobilised on high binding
163 ELISA plates (Greiner Bio-One Ltd, UK) overnight at 4°C. EVs were blocked for 2 hours at room
164 temperature using 1% (w/v) BSA before adding 3 µg/mL primary antibodies of interest (anti-CD41,
165 anti-CD11b, anti-CD144 and anti-CD235a; as detailed above) in 0.1% (w/v) BSA overnight at
166 4°C. Primary antibodies were detected using a biotin-labeled goat anti-rabbit IgG secondary antibody⁴¹
167 (diluted 1:2500 in 0.1% BSA, purchased from Perkin Elmer, UK) for 1 hour at room temperature,
168 followed by a streptavidin-europium conjugate (diluted 1:1000 in red assay buffer, both Perkin Elmer)
169 for 45 minutes at room temperature. Time resolved fluorescence was measured on a BMG
170 CLARIOstar® plate reader (BMG Labtech, UK).

171

172 *Detection of an array of adipokines in plasma EV samples*

173 A commercially available Proteome Profiler Human Adipokine Array Kit (R&D Systems, Bio-
174 Techne, UK) was used to analyse 58 adipocyte-related molecules in pre-depletion, post-magnetic
175 bead depletion and post-solid phase depletion EV samples. Samples were diluted to load an absolute
176 concentration of 2×10^{10} particles. The remainder of the experiment was performed according to the
177 manufacturer's protocol. Dot assays were detected using Amersham ECL Hyperfilm following 15-
178 and 60-minute exposures. Blots were scanned and pixel densities analysed using HLIImage++
179 (Western Vision Software, USA). A full list of analytes included in the kit is shown in Table S1.

180

181 *Statistical analysis*

182 Data are presented as mean \pm SEM. A one-way ANOVA with Tukey's Multiple Comparison Test was
183 used to analyse the difference between means. A p value of <0.05 was considered significant. Data
184 were analysed using Graph Pad Prism (version 5; GraphPad Software Inc., CA).

185

186 **Results**

187 *Preparation of plasma-derived EVs using size exclusion chromatography*

188 Analysis of individual column fractions using Nanoparticle Tracking Analysis (NTA) showed a small
189 peak in the concentration of particles/mL between fractions 5-10, followed by a large peak in particles
190 and protein from fractions 12-26. Western blot analysis of fractions 2-28 showed the presence of both
191 EV and adipocyte markers in fractions 6-10 but only adipocyte markers in fractions 11-28 (Figure
192 S1). Plotting the ratio of particle concentration to protein concentration as described previously⁴²
193 showed fractions 5-10 to contain the highest number of particles:protein (Figure 1A). Therefore, these
194 fractions were pooled and ultracentrifuged to pellet plasma-derived EVs. TEM of pelleted EVs
195 indicated the presence of vesicle structures, and Western blot analysis showed the presence of
196 classical EV and adipocyte markers in the pooled EVs of three different individuals (Figure 1B/C).
197 Pooled EVs were shown to be deficient in the endoplasmic reticulum marker, Grp-94 (Figure S2A), in
198 accordance with ISEV guidelines for expected proteins in EV isolates¹³. The supernatant of pelleted
199 EVs following ultracentrifugation was deficient in CD9 (Figure S2B) indicating EVs were
200 successfully pelleted by ultracentrifugation.

201

202 *Adipocyte markers remain following sequential depletion of major EV families*

203 Magnetic beads and a solid phase-based method were used to sequentially deplete EVs bearing
204 markers of the four major EV populations in the circulating plasma of three different individuals.
205 TEM analysis revealed EV structures to be present in both post-magnetic bead and post-solid phase
206 depletion samples (Figure 2A). EV concentration was reduced by ~75% in both post-magnetic bead
207 and post-solid phase depletion samples: $1.01 \times 10^{11} \pm 1.00 \times 10^{10}$ particles/mL to $3.10 \times 10^{10} \pm 6.90 \times$
208 10^9 particles/mL and $2.50 \times 10^{10} \pm 6.50 \times 10^9$ particles/mL respectively, $p < 0.001$, (n=5); Figure 2B.
209 The detection of markers of the main EV populations in plasma (Platelet; CD41, monocytes; CD11b,
210 endothelial cells; CD144 and erythrocytes; CD235a) were reduced in post-depletion samples
211 following magnetic bead and solid-phase-based methods (Figure S3). Adiponectin, FABP4, PPAR γ ,

212 Perilipin, CD9 and Alix were reduced but still detectable in post-magnetic bead and post-solid phase
213 depletion samples (Figure 2C). Interestingly, only the adipocyte specific PPAR γ -2 isoform remained
214 in post-depletion samples.

215

216 *Major adipokines are expressed in pre and post-depletion samples*

217 An adipokine array kit was used to probe for 58 adipokines (Table S1) in pre-depletion, post-magnetic
218 bead and post-solid phase depletion plasma EV samples (Figure 3A). Major adipokines, including
219 adiponectin, adipsin, leptin, preadipocyte factor (PREF)-1, resistin and visfatin, were detected in all
220 samples (Figure 3B). No significant differences were observed between samples.

221

222 **Discussion**

223 This study is the first of its kind to present a variety of evidence for the presence of adipocyte-derived
224 EVs in the circulating plasma of healthy individuals. A panel of adipocyte markers and adipokines
225 were detected in plasma EV samples after careful sample processing and depletion of EVs from major
226 circulating sources. Adipocyte-derived EVs have proven to be important, novel endocrine mediators
227 of adipocytes *in vitro*, thus their detection in the human circulation is an important step towards
228 understanding their roles as mediators of adipocyte function, including potential effects on vascular
229 health.

230 Due to the complexity of plasma as a biofluid, platelet-depleted plasma was loaded onto size
231 exclusion chromatography (SEC) columns. SEC has previously been shown to separate EVs quickly
232 and effectively from the majority of non-vesicular protein in plasma^{39,43}. Here, EVs were identified in
233 fractions 5-10 from the high particle-to-protein ratio and the presence of EV markers, CD9, CD81 and
234 Alix in these fractions (Figure 1 and Figure S1). Later fractions had a low particle-to-protein ratio and
235 EV markers were not identified in these fractions. Additionally, the adipocyte markers adiponectin,
236 FABP4, Perilipin and PPAR γ were detected in fractions 5-10 but were also present in later fractions.
237 Detection of these markers is in keeping with previous studies that have identified adipocyte markers
238 within EVs from human plasma^{4,11,18,22}. However, our data indicates that markers previously used to
239 identify adipocyte-derived EVs in un-purified plasma samples are largely soluble and likely not
240 associated with EVs as illustrated in Figure S1, where we show adiponectin, FABP4, Perilipin and
241 PPAR γ are all detected as soluble protein in SEC fractions not containing EVs, despite loading up to
242 55x less volume. This finding has important implications for the measurement of adipocyte EV
243 markers in human plasma, and highlights the importance of techniques such as SEC prior to analysis
244 of adipocyte markers to avoid erroneous overestimations from soluble material. Pooling and
245 subsequent ultracentrifugation of these fractions confirmed the presence of EV structures by TEM and
246 both EV and adipocyte proteins by Western blotting (Figure 1, Figure S2A/B). We also observed the
247 presence of adipokines in the supernatant of pelleted EVs highlighting the importance of the
248 ultracentrifugation step after SEC. This is in keeping with previous studies, which have shown that

249 SEC is effective in removing ~95% of non-vesicular protein in a single step, but the EV-free
250 supernatant is likely to contain residual, non-EV-associated plasma proteins, including adipokines^{39,43}.

251 The majority of plasma-derived EVs originate from cells that are in direct contact with blood, such as
252 platelets, leukocytes, vascular endothelial cells and erythrocytes⁴⁴. The location of adipocytes within
253 adipose tissue may hinder the majority of adipocyte-derived EVs reaching the systemic circulation.
254 Consequently, adipocyte-derived EVs are likely to form only a minor proportion of plasma-derived
255 EVs. Furthermore, markers that uniquely identify adipocytes, such as adiponectin are readily secreted.
256 High-speed centrifugation used for EV isolation may co-pellet these soluble markers with EVs,
257 lending a false adipocyte character. We therefore applied two separate techniques to deplete the major
258 circulating populations of plasma-derived EVs to establish whether adipocyte markers were reduced
259 by depletion of “non-adipocyte” EVs and whether an adipocyte protein signature was retained post-
260 depletion. EV structures were visible by TEM following sequential depletion of major plasma EV
261 populations (Figure 2A) though the overall concentration of EVs detected by NTA was reduced by
262 around 75% (Figure 2B). Both techniques were shown to reduce the expression of each marker used
263 for depletion, with the magnetic bead based approach depleting these markers beyond detection by
264 time resolved fluorescence (Figure S3). This suggests both techniques are effective in reducing the
265 populations of major circulating EVs in plasma. Expression of adiponectin, FABP4 and PPAR γ was
266 reduced post-depletion (Figure 2C), suggesting a proportion of these markers are in some way
267 associated with EVs from non-adipocyte populations. Although their expression is predominantly
268 associated with adipocytes, both FABP4 and PPAR γ have previously been shown to be produced by
269 other cells including macrophages^{45,46} perhaps explaining the partial loss in signal post-depletion.

270 FABP4 was not detected in all samples (possibly due to individual variations in donors) and was often
271 detected at a higher molecular weight than expected. FABP4 has previously been reported to form
272 homodimers, particularly upon ligand activation⁴⁷ though the absence of expression in some samples
273 reaffirms the need to use multiple markers when analysing adipocyte-derived EVs. However, whilst
274 both isoforms of PPAR γ were detected in pre-depletion samples, only PPAR γ 2 remained in post-
275 depletion samples. PPAR γ 2 is an adipocyte-specific nuclear transcription factor⁴⁸ and its presence in

276 combination with adiponectin, FABP4 and Perilipin in post-depletion samples is highly indicative of
277 adipocyte origin. Furthermore, a number of major adipokines were detected in post-depletion samples
278 using an adipokine array kit, including the angiogenic factors leptin and resistin, and adipokines
279 adipsin, PREF-1, and visfatin (Figure 3). This further evidences the presence of adipocyte markers in
280 EV samples that have been depleted of major circulating plasma EV populations. The EV markers
281 Alix and CD9 were reduced but still present in post-depletion samples (Figure 2C). This tallies with a
282 reduced concentration of EVs but also indicates that EVs are still present in post-depletion samples,
283 supporting the TEM data. Taken together, our data show that after depleting EVs from major sources
284 in plasma using either magnetic beads or solid phase depletion, adipocyte and EV markers are still
285 detectable, supporting the presence of adipocyte-derived EVs. It is important to note that we observed
286 differences in expression patterns of both EV and adipocyte markers between individuals, however,
287 this is most likely due to natural biological variation within our small group of donors.

288 In conclusion, this study is the first of its kind to provide evidence for the presence of adipocyte-
289 derived EVs in circulating plasma using multiple adipocyte and EV markers, and conducted in
290 accordance with international recommendations. Our data also emphasize the need for careful EV
291 preparation when analysing adipocyte markers to avoid contribution of signal from soluble adipocyte
292 material. Whilst adipocyte-derived EVs may only constitute a relatively small fraction of the total EV
293 population in circulating plasma, this may not necessarily reflect a minor effect on vascular health
294 since the content of the EVs is likely to dictate their function, particularly in EVs derived from
295 dysfunctional adipocytes. Our data thus provide a platform for future investigations into circulating
296 adipocyte-derived EVs as potential novel biomarkers of adipocytes in health and obesity-driven
297 cardiovascular disease.

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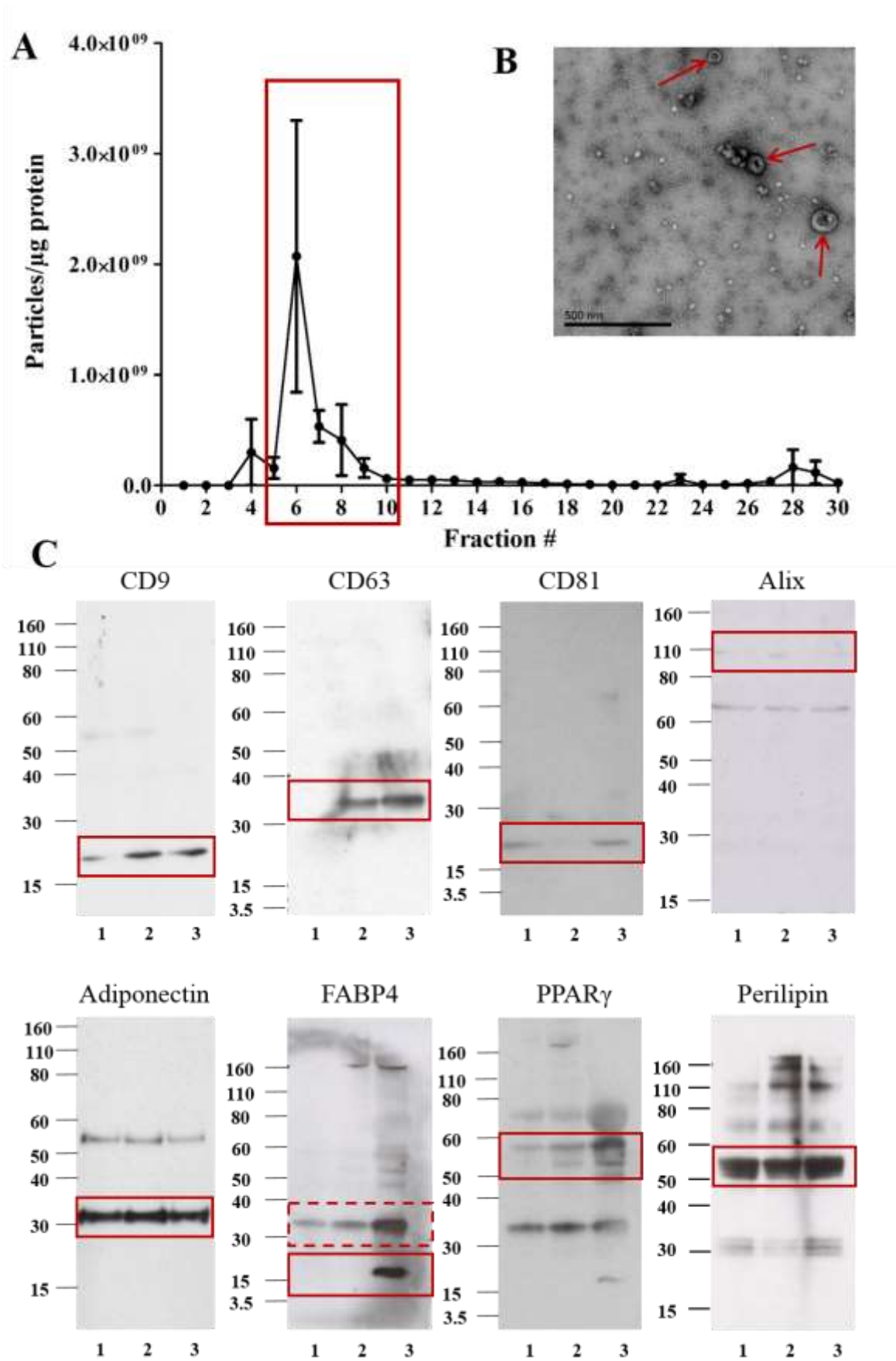
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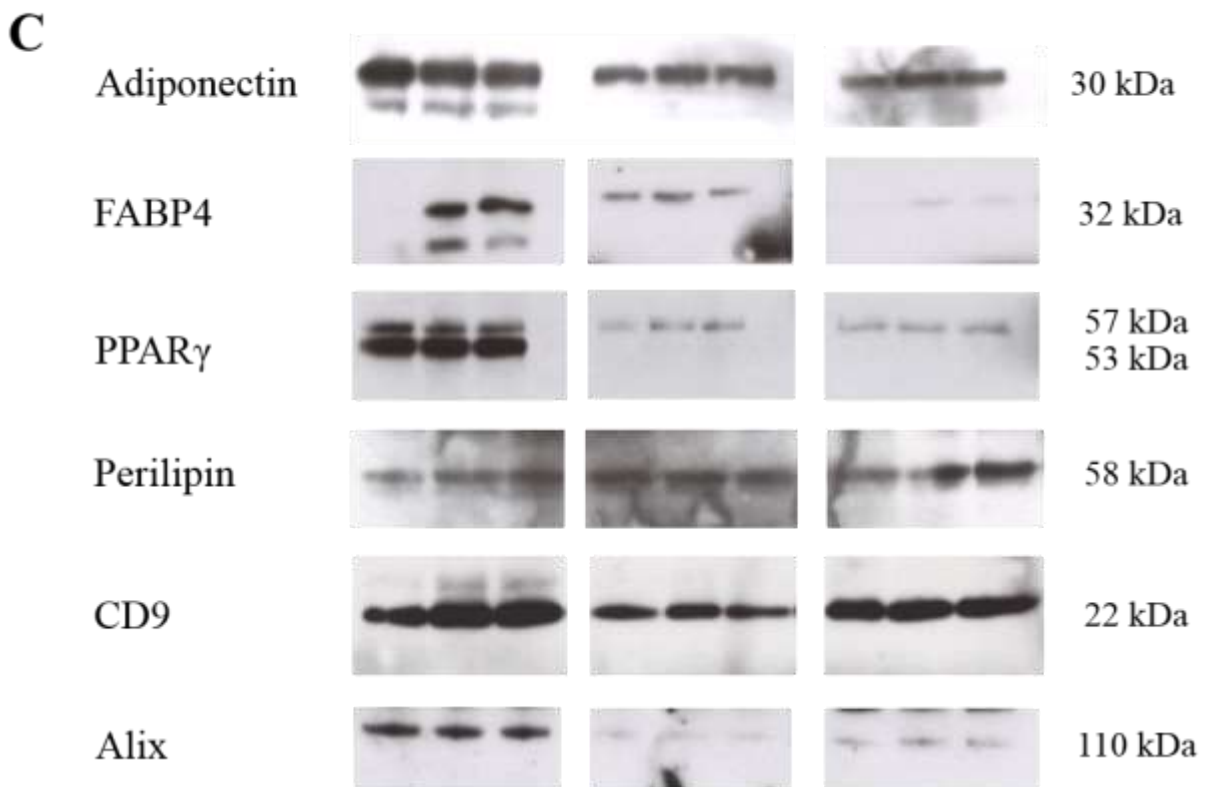
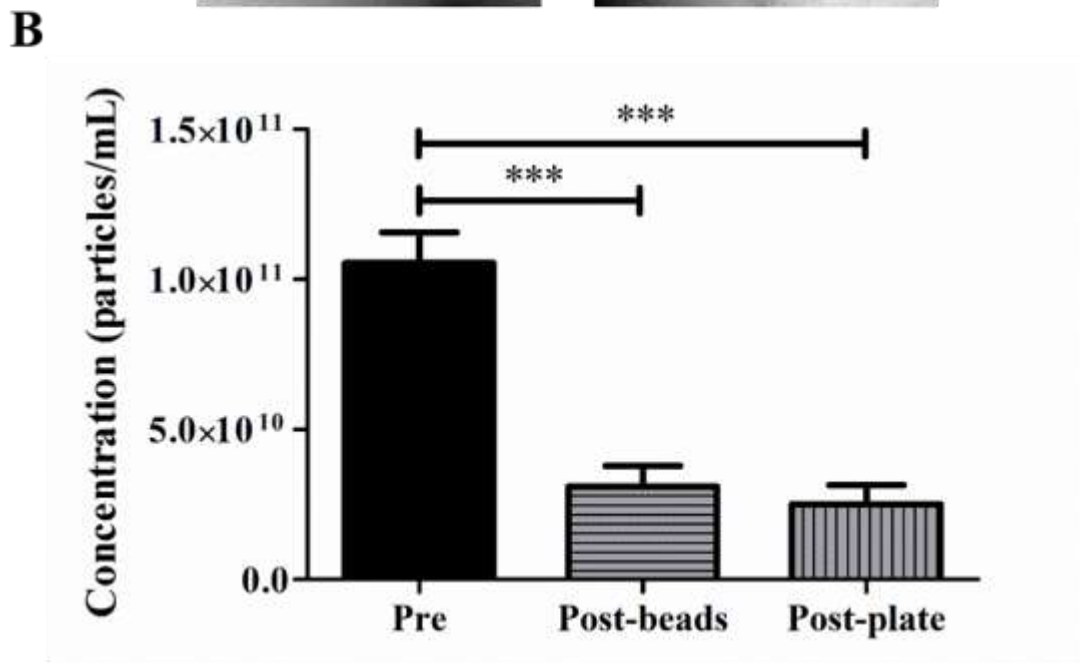
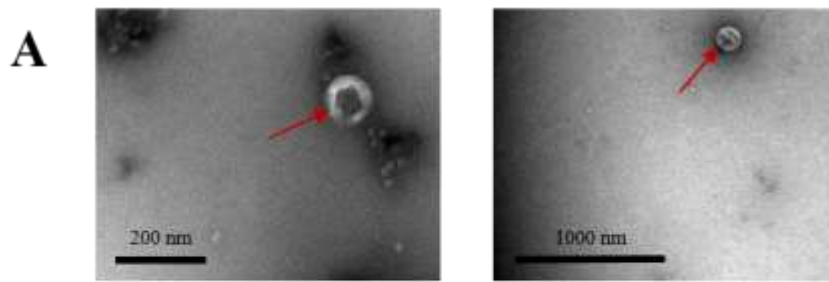
416 **Figure 1: Detailed analysis of pooled plasma EVs.** (A) Fractions 5-10 showed the highest ratio of
417 particles-to-protein and the presence of EV structures by TEM (B, red arrows indicate EV structures)
418 following ultracentrifugation. (C) Pooled EVs from three different individuals (labelled 1, 2, and 3)
419 were analysed by Western blot for EV markers: CD9, CD63, CD81 and Alix, and adipocyte markers:
420 Adiponectin, FABP4, PPAR γ and Perilipin (n=3). Solid red boxes indicate the predicted molecular
421 weight for each antigen; the dotted red box may indicate a FABP4 dimer ~32 kDa.

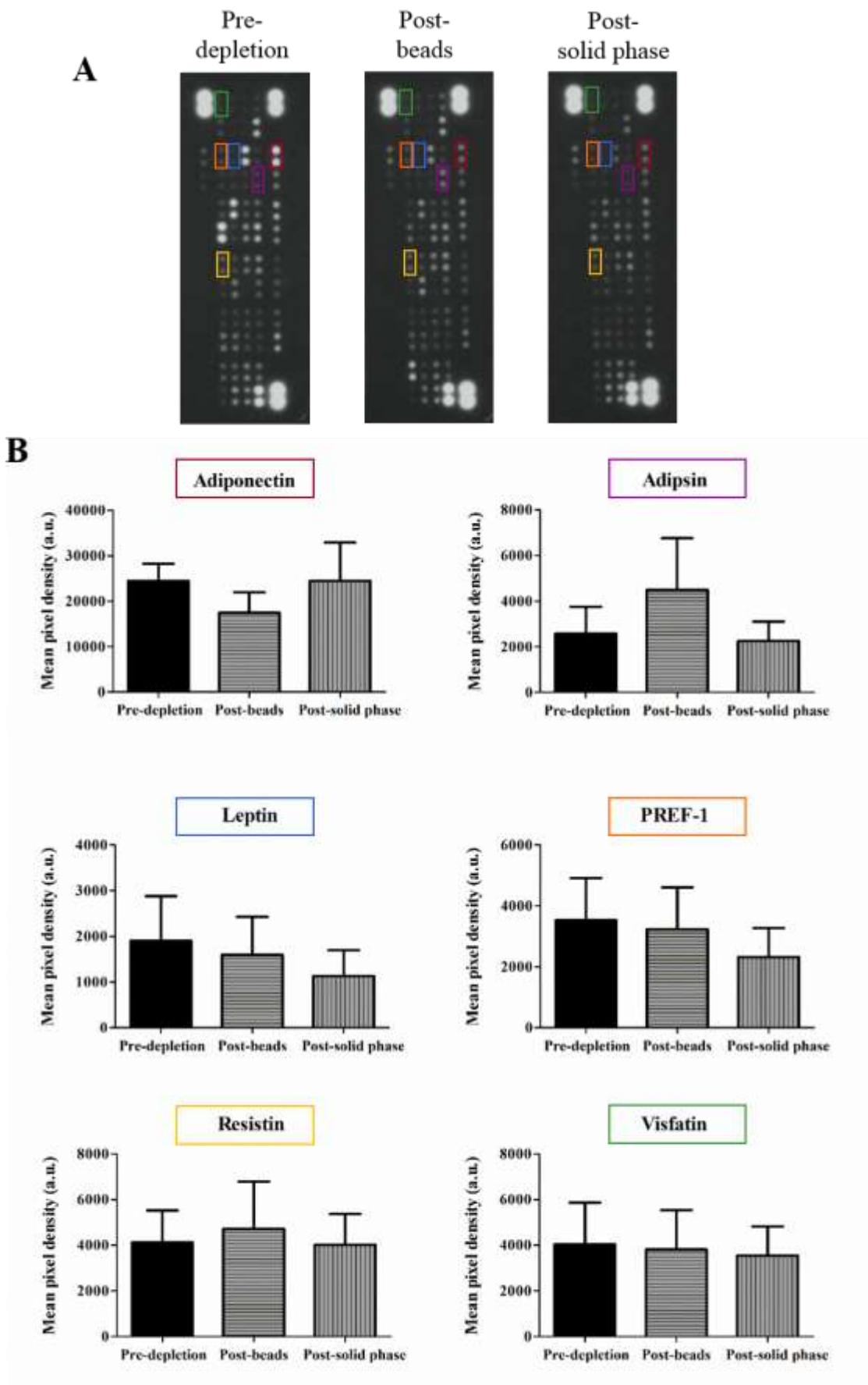
422 **Figure 2: Adipocyte and EV markers were maintained post-magnetic bead and solid phase**
423 **depletion.** (A) EV structures were visible by TEM in post-magnetic bead depletion (left, scale bar 200
424 nm) and post-solid phase depletion (right, scale bar 1000 nm). (B) EV concentration was reduced
425 following sequential depletion of major EV families using magnetic beads or a solid phase method,
426 *** $p = 0.005$ (n=5). (C) Adiponectin, FABP4, PPAR γ -2, Perilipin, CD9 and Alix were still present in
427 post-depletion samples of three different individuals.

428 **Figure 3: Major adipokines were present in post-magnetic bead and –solid phase depletion**
429 **samples.** (A) Inverted raw data of dot blots pre-depletion, post-magnetic beads and post-solid phase
430 depletion. Major adipokines are highlighted with corresponding pixel densities (B). Representative
431 dot blots of n=3.

432







439 **Supplemental data**

440 **Figure S1: Co-elution of EV and adipocyte markers from human plasma.** Plasma (1 mL) was
441 loaded onto SEC columns and 30 x 500 μ L fractions were collected. The concentration of
442 particles/mL was measured using NTA and the protein concentration was measured using Nanodrop
443 for each fraction. Fractions 2 – 28 were then analysed by Western blot (8 μ g/lane) for the EV
444 markers; CD9, CD81 and Alix, and the adipocyte markers; Adiponectin, FABP4, Perilipin and
445 PPAR γ . A small peak in particle concentration in fractions 5-10 corresponds with the detection of EV
446 and adipocyte markers. A larger peak in particles and protein in fractions 12-26 corresponds with
447 adipocyte markers only (n=3).

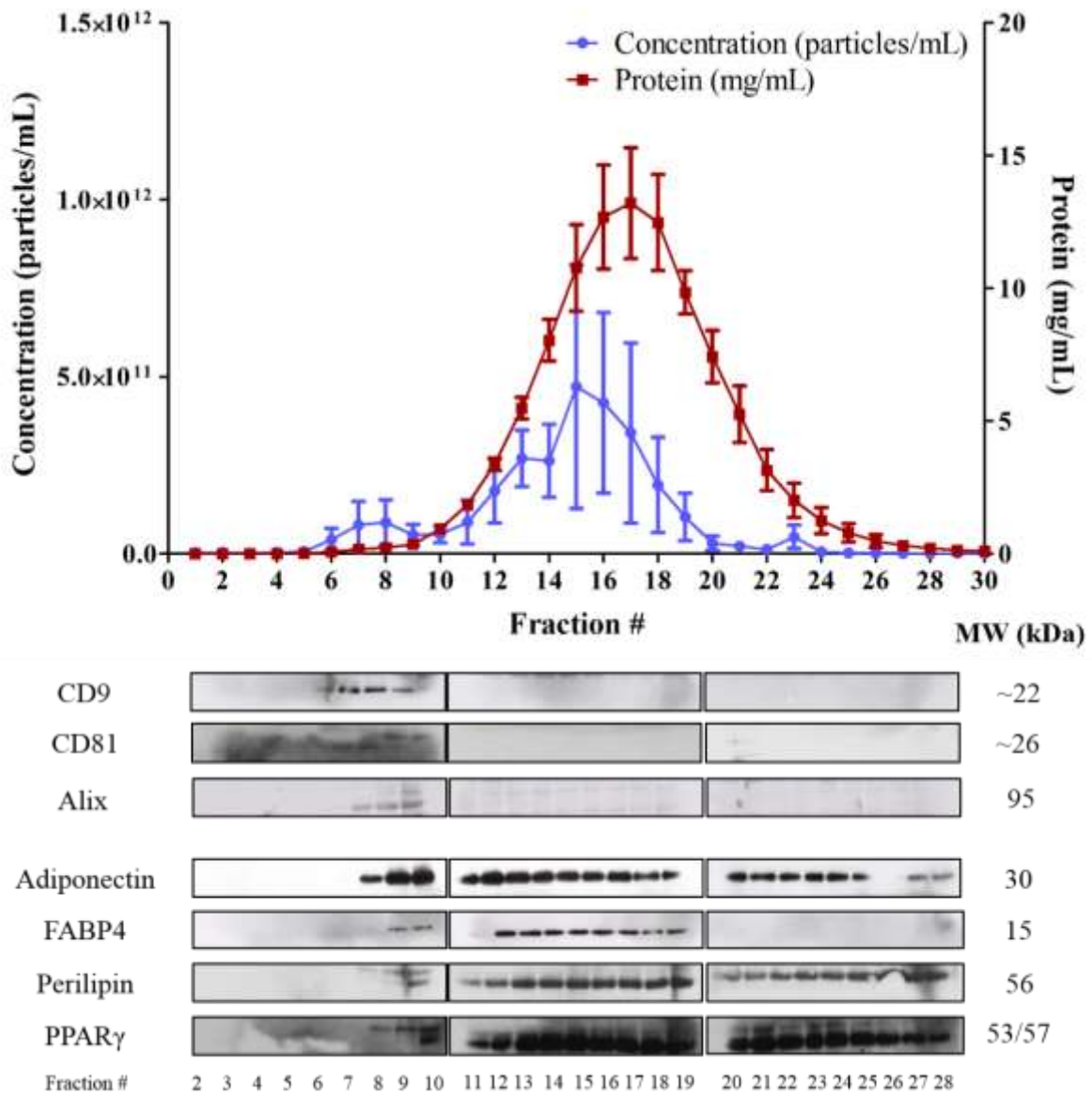
448 **Figure S2: Confirmation of an EV population.** (A) Western blot analysis of 3T3-L1 cell (positive
449 control to confirm antibody specificity) and pooled plasma EV lysates for the endoplasmic reticulum
450 protein, Grp-94 (MW~100 kDa). (B) Western blot analysis of pooled plasma EVs and the
451 corresponding supernatant from the EV pellet following ultracentrifugation for the EV marker, CD9
452 (MW~ 22kDa).

453 **Figure S3: The efficiency of magnetic bead and solid phase depletion.** Equal numbers of EVs were
454 immobilised onto ELISA plates pre- and post-CD41, -CD11b, -CD144 and -CD235a depletion using
455 magnetic beads (A) and solid phase (B). Pre and post samples were then analysed by time resolved
456 fluorescence for the presence of the depleted marker and plotted as a percentage of the “Pre” sample
457 fluorescence. ND = not detected.

458 **Table S1: Adipokine array appendix.** A list of the 58 analytes included in adipokine array kit with
459 mean pixel density values \pm SEM for pre-depletion, post-magnetic bead depletion and post-solid
460 phase depletion samples (n=3).

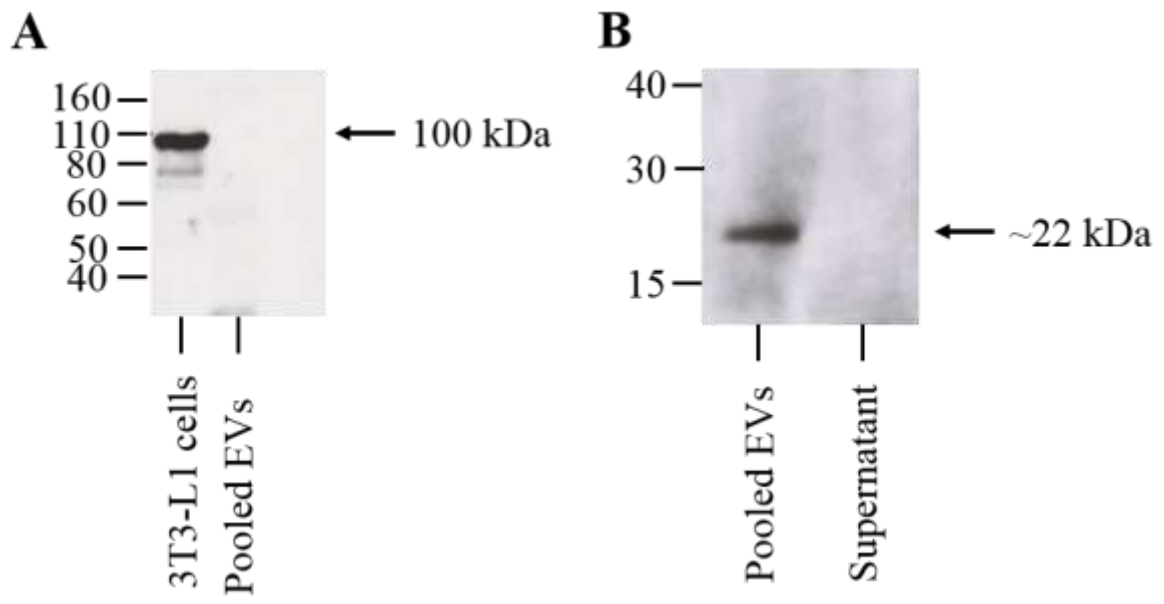
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462 **Supplementary Figure 1**



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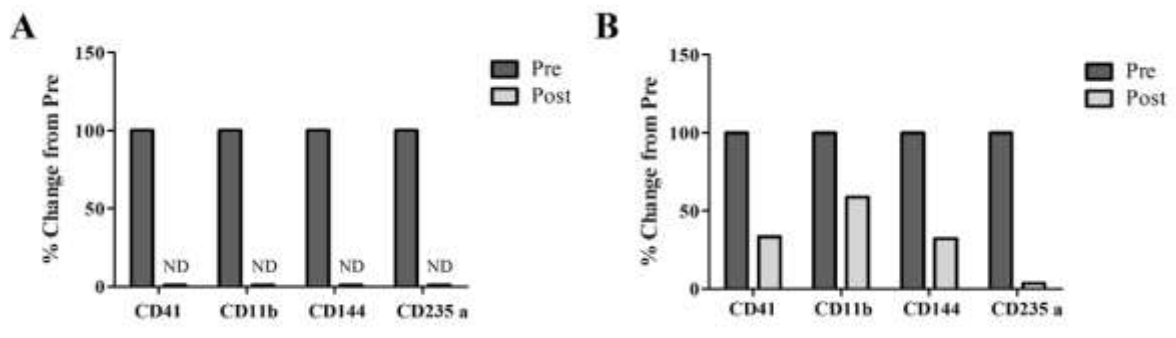
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468 **Supplementary Figure 3**



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471 **Supplementary Table 1**

Adipokine	Pre-depletion	Post-beads	Post-solid phase
Adiponectin	24,425 ± 3,808	17,417 ± 4,531	24, 429 ± 8,412
Angiopoietin-1	5,978 ± 2,030	4,961 ± 1,892	5,465 ± 2,071
Angiopoietin-2	9,963 ± 2,596	8,095 ± 2,270	7,482 ± 2,509
Angiopoietin-like 2	7,484 ± 1,997	6,260 ± 2,090	5,543 ± 2,023
Angiopoietin-like 3	2,153 ± 908	2,582 ± 1,075	1,772 ± 721
CD257 (B cell activating factor)	3,182 ± 1,165	3,212 ± 1,280	2,584 ± 933
Bone morphogenetic protein (BMP)-4	3,403 ± 1,269	3,267 ± 1,392	3,565 ± 1,212
Cathepsin D	8,320 ± 2,891	7,734 ± 3,011	11,028 ± 5,763
Cathepsin L	3,310 ± 1,690	9,065 ± 3,214	2,661 ± 1,404
Cathepsin S	13,899 ± 4,093	13,172 ± 4,106	11,031 ± 4,783
Chemerin	2,000 ± 983	2,405 ± 1,119	1,964 ± 790
Complement Factor D (Adipsin)	2,578 ± 1,173	4,494 ± 2,262	2,254 ± 851
C-Reactive Protein (CRP)	8,585 ± 3,518	6,317 ± 1,873	8,281 ± 3,709
Dipeptidyl peptidase (DPP)-4	9,591 ± 3,337	11,138 ± 3,757	10,306 ± 4,681
Endocan	7,882 ± 2,761	9,140 ± 3,097	7,221 ± 2,227
EN-RAGE	3,846 ± 1,619	4,634 ± 1,763	3,033 ± 1,110
Fetuin B	2,620 ± 964	3,060 ± 1,265	2,104 ± 659
Fibroblast Growth Factor (FGF)-2	2,407 ± 921	2,355 ± 1,156	1,624 ± 426
FGF-19	6,987 ± 2,361	5,403 ± 2,117	7,197 ± 2,920
Fibrinogen	35,002 ± 4,995	38,828 ± 2,134	36,146 ± 5,145
Growth Hormone	2,314 ± 571	3,279 ± 738	2,149 ± 62
Hepatocyte growth factor (HGF)	1,932 ± 382	2,340 ± 402	1,210 ± 604
Intercellular adhesion molecule (ICAM)-1	11,422 ± 5,785	8,230 ± 1,496	7,768 ± 1,746
Insulin growth factor binding protein (IGFBP)-2	3,047 ± 290	2,243 ± 881	1,313 ± 603
IGFBP-3	4,233 ± 1,710	3,945 ± 1,320	3,489 ± 1,225

IGFBP-4	7,911 ± 2,475	8,848 ± 3,219	6,903 ± 2,286
IGFBP-6	5,850 ± 2,075	7,141 ± 2,633	5,144 ± 1,663
IGFBP-7	1,206 ± 185	1,451 ± 20	527 ± 398
Interleukin (IL)-1 β	2,921 ± 930	3,149 ± 1,269	2,447 ± 891
IL-6	3,234 ± 1,039	3,238 ± 1,417	2,919 ± 1,260
IL-8	7,350 ± 2,172	7,811 ± 2,729	8,001 ± 3,149
IL-10	8,716 ± 3,176	8,237 ± 3,133	10,025 ± 3,603
IL-11	1,928 ± 892	2,622 ± 1,351	1,779 ± 823
Transforming growth factor (TGF)- β 1	1,687 ± 282	2,024 ± 327	1,601 ± 34
Leptin	1,902 ± 978	1,596 ± 832	1,135 ± 567
Leukaemia inhibitory factor (LIF)	2,310 ± 1,056	2,055 ± 906	1,704 ± 748
Lipocalin-2	26,197 ± 2,855	24,184 ± 7,044	24,200 ± 8,432
Monocyte chemoattractant protein (MCP)-1	2,640 ± 676	2,864 ± 1,047	2,464 ± 430
Macrophage colony stimulating factor (M-CSF)	2,847 ± 1,034	3,270 ± 1,435	2,161 ± 709
Macrophage migration inhibitory factor (MIF)	4,646 ± 1,785	12,566 ± 2,293	4,130 ± 1,121
Myeloperoxidase	2,035 ± 641	2,378 ± 1,054	1,727 ± 503
Nidogen-1	4,820 ± 1,340	7,034 ± 2,488	4,173 ± 1,853
Oncostatin M	4,984 ± 1,743	3,647 ± 1,555	4,961 ± 2,042
Pappalysin-1	8,222 ± 2,764	5,699 ± 2,099	8,715 ± 3,445
Pentraxin-3	3,903 ± 1,809	3,639 ± 1,703	3,401 ± 1,608
Preadipocyte factor (PREF)-1	3,525 ± 1,393	3,237 ± 1,378	2,325 ± 943
Proprotein convertase 9	1,953 ± 937	1,904 ± 969	1,423 ± 706
RAGE	3,676 ± 663	3,041 ± 1,251	3,194 ± 1,202
CCL5	26495 ± 6,371	15,406 ± 4,790	25,518 ± 8,363
Resistin	4,121 ± 2,436	4,731 ± 2,062	4,024 ± 1,348
Serpin A8	1,712 ± 639	1,979 ± 826	1,437 ± 499
Serpin A12	2,215 ± 128	1,997 ± 990	1,327 ± 329

Plasminogen activator inhibitor (PAI)-1	5,613 ± 1,623	4,858 ± 1,909	4,560 ± 1,853
Tissue inhibitor of metalloproteinase (TIMP)-1	4,024 ± 1,006	24,028 ± 4,264	3,550 ± 1,231
TIMP-3	1,932 ± 390	1,015 ± 352	1,557 ± 690
Tumor necrosis factor (TNF)- α	4,260 ± 1,574	5,900 ± 2,245	4,942 ± 1,872
Vascular endothelial growth factor (VEGF)	2,338 ± 160	2,669 ± 1,105	1,555 ± 716
Visfatin	4,029 ± 1,842	3,820 ± 1,715	3,540 ± 1,285

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