1 **Young women with polycystic ovary syndrome have raised levels of circulating annexin**
2 **V-positive platelet microparticles**

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9 **Running title:** Blood microparticles and polycystic ovary syndrome

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12

13 **Abstract**

14 **Study question:** Are circulating microparticles (MPs) altered in young women with
15 polycystic ovary syndrome (PCOS)?

16 **Summary answer:** Women with PCOS have elevated concentrations of circulating platelet-
17 derived MPs, which exhibit increased annexin V binding and altered microRNA (miR)
18 profiles compared to healthy volunteers.

19 **What is known already:** Some studies have shown that cardiovascular risk is increased in
20 young women with PCOS but the mechanisms by which this occurs is uncertain. Circulating
21 MPs are elevated in patients with cardiovascular disease but the characteristics of MPs in
22 patients with PCOS are unclear.

23 **Study design**: Case-control study comprising 17 women with PCOS (Mean ± SD; age 31 ± 7
24 yrs, BMI 29 ± 6 kg/m^2) and 18 healthy volunteers (age 31 ± 6 yrs, BMI 30 ± 6 kg/m^2).

25 **Participants/materials, setting, methods:** The study was conducted in a University hospital.
26 Nanoparticle tracking analysis and flow cytometry (CD41 platelet, CD11b monocyte, CD144
27 endothelial) were used to determine MP size, concentration, cellular origin and annexin V
28 positivity (reflecting phosphatidylserine exposure). Fatty acid analysis was performed by gas
29 chromatography and MP miR expression profiles were compared by microarray.

30 **Main results and the role of chance:** PCOS subjects showed increased MP concentrations
31 compared to healthy volunteers (Mean ± SD; 11.5 ± 5 x10^{12}/ml versus 10.0 ± 4 x10^{12}/ml,
32 respectively; p = 0.03), which correlated with the homeostasis model of insulin resistance
33 (r=0.53, p=0.03). This difference was predominantly seen in MPs whose size was in the small
34 exosomal range (<150 nm in diameter, p <0.05). PCOS patients showed a greater percentage
35 of annexin V$^+$ MPs compared to healthy volunteers (84 ± 18 % versus 74 ± 24 %,
36 respectively, p = 0.05) but the cellular origin of MPs, which were predominantly platelet-

37 derived (PCOS: 99 ± 0.9%; controls: 99 ± 2.5%), did not differ. MP fatty acid concentration
38 and composition was similar between groups but 16 miRs were differentially expressed
39 ($p<0.05$).

40 **Limitations, reason for caution:** Patients with PCOS were classified by the Rotterdam
41 criteria, which describes a less severe metabolic phenotype than other definitions of the
42 syndrome. Our findings may thus not be generalisable to all patients with PCOS. MicroRNA
43 expression analysis was only undertaken in an exploratory subset of the overall study
44 population, hence validation of our findings in a larger cohort is mandatory. Furthermore,
45 miR levels were unaltered for the highly expressed miRs and it is unclear whether differences
46 in the lowly expressed miRs carries pathological relevance.

47 **Wider implications of the findings:** This study suggests that women with PCOS have an
48 altered MP profile but further studies are needed to confirm this, to explore the mechanisms
49 by which these alterations develop and to establish whether therapies that improve insulin
50 sensitivity are able to reduce circulating MP concentrations.

51 **Study funding/competing interest(s):** The study was funded by grants from the Wales Heart
52 Research Institute and Mrs John Nixon Scholarship. The authors have no conflicts of interest
53 to declare.

54 **Key words:** Polycystic Ovary Syndrome, microparticles, insulin resistance.

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60 **Introduction**

61 Polycystic ovary syndrome (PCOS) is a common endocrine condition characterised by
62 hyperandrogenism, polycystic ovaries and oligo/anovulation. In addition to its reproductive
63 sequelae, PCOS is now considered a metabolic disorder characterised by defects in insulin
64 secretion and sensitivity (Ehrmann *et al*., 1995), which lead to an increased risk of type 2
65 diabetes (Morgan *et al*., 2012). Patients may also be at increased risk of cardiovascular
66 disease but the mechanisms by which these occur are not yet fully established. One process
67 may involve endothelial dysfunction (El-Kannishy *et al*., 2010, Orio *et al.,* 2004), an early
68 marker of vascular disease which is associated with reduced nitric oxide (NO) bioavailability,
69 increased oxidative stress and elevated circulating microparticles (MPs) (Amabile *et al.*,
70 2005, Gündüz *et al.,* 2012).

71 MPs are small (30-1000 nm diameter) membrane-enclosed vesicles released from a variety of
72 eukaryotic and prokaryotic cells including platelets, monocytes and endothelial cells (van der
73 Pol *et al*., 2012). They represent a homeostatic communication network between source and
74 target cells, but may also play a role in disease pathology. Marked elevations in MP
75 concentration have been reported in patients with cancer (Kim *et al.*, 2003), diabetes (Koga *et*
76 *al.,* 2005), sepsis (Nieuwland *et al.,* 2000), hypertension (Preston *et al*., 2003) and myocardial
77 ischaemia (Boulanger *et al*., 2001). Furthermore, elevations in platelet-derived MPs (PMPs)
78 have been observed in patients with coronary artery disease (CAD) (Koga *et al.*, 2005, Mallat
79 *et al.*, 2000).

80 These observations suggest that MPs may play a role in the pathogenesis of vascular
81 dysfunction in 'at risk' populations, but the characteristics of circulating MPs in patients with
82 PCOS are poorly described. Koiou *et al*., (2011) reported increased PMP concentrations in
83 patients with hyperandrogenic PCOS, but the MP cell-of-origin, fatty acid composition and
84 cellular cargo were not assessed in their study. In light of these considerations, we sought to
85 undertake a detailed characterisation of circulating MP populations in patients with PCOS.

86 **Methods**

87

88 **Subjects and protocol**

89 Seventeen PCOS patients (age 16-45 years) were recruited from the endocrine clinic at the
90 University Hospital of Wales (UHW). PCOS was diagnosed according to the Rotterdam
91 criteria. Congenital adrenal hyperplasia, Cushing's syndrome, hyperprolactinaemia,
92 androgen-secreting tumours and thyroid disease were excluded by biochemical testing.
93 Subjects were excluded from participation if they were pregnant, breastfeeding or had a
94 history of hypertension, hyperlipidaemia or diabetes. Additional exclusion criteria included a
95 history of current or recent (within 3 months) use of antidiabetics, lipid-lowering agents,
96 antihypertensives and/or antiandrogens. Eighteen healthy volunteers (age 16-45 years) were
97 recruited among medical students and staff within our institution. Healthy controls had
98 regular menstrual cycles (every 27-32 days). Their healthy state was established by history,
99 physical examination and hormonal evaluation (thyroid function, prolactin, testosterone and
100 17-hydroxyprogesterone); those with features of hirsutism or a family history of PCOS were
101 excluded. The study was approved by Cardiff University (study sponsors), Cardiff & Vale
102 University Health Board and the South East Wales Research Ethics Committee. All subjects
103 gave written informed consent before study commencement.

104

105 **Anthropometric and biochemical measurements**

106 Subjects attended our Clinical Research Facility at 0800h after an overnight fast. Studies were
107 conducted in a quiet, temperature-controlled room and subjects were required to rest for 10
108 minutes before study measurements. Height, weight, hip and waist circumference were
109 measured as per our published protocols (Watson *et al*., 2009). Serum total cholesterol (TC),
110 high density lipoprotein cholesterol (HDL), and triglycerides (TG) were assayed using an
111 Aeroset automated analyser (Abbott Diagnostics, Berkshire, UK); low density lipoprotein

112 cholesterol (LDL) was calculated using Friedewald's formula. Insulin was measured using an
113 immunometric assay specific for human insulin (Invitron, Monmouth, UK) and glucose was
114 measured using the Aeroset chemistry system (Abbott Diagnostics, Berkshire, UK). High
115 sensitivity C-reactive protein (hsCRP) was assayed by nephelometry (BN™ II system; Dade-
116 Behring, Milton Keynes, UK) and total testosterone was measured by liquid chromatography-
117 tandem mass spectrometry (Quattro™ Premier XE triple quadrupole tandem mass
118 spectrometer; Waters Ltd, Watford, UK). The intra- and inter-assay coefficients of variation
119 were all less than 9%. After basal sampling, subjects underwent a standard 75g oral glucose
120 tolerance test (OGTT). Glucose and insulin were measured at 0, 30, 60, 90 and 120 minutes.
121 The area under the curve (AUC) for insulin and glucose was calculated using the trapezoid
122 method. Fasting insulin resistance was also estimated by the homeostasis model assessment
123 method (HOMA-IR).

124

125 **Blood sampling, isolation and storage of microparticles**

126 Fasting blood samples were drawn from an antecubital vein into ethylenediaminetetraacetic
127 acid vacutainers. Blood samples were promptly centrifuged (1,024*g* x 10min at 4 °C) to yield
128 platelet-poor plasma. Plasma-derived MPs were isolated via differential ultracentrifugation.
129 Briefly, plasma (1 ml) was ultracentrifuged (100,000*g* x 1 hr at 4 °C; Beckman Coulter, UK)
130 and the supernatant was discarded, as previously described (Connolly *et al*., 2014). The
131 remaining pellet was resuspended in 250 µl of RNAase-free phosphate-buffered saline (Fisher
132 Scientific, UK) which had been filtered using a 0.22 µm Millipore (Merck Millipore, UK).
133 Isolated MPs were stored at -80 °C, for no longer than 6 months before analysis. For use,
134 samples were thawed in a preheated (37 °C) thermostatically-regulated water bath for 3
135 minutes.

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137

138 **Nanoparticle tracking analysis (NTA)**

139 MP size and concentration were determined using nanoparticle tracking analysis (NTA)
140 (NanoSight LM10 system, UK) as described previously (Webber, 2013). Briefly, NTA is a
141 laser illuminated microscopic technique equipped with a 405 nm laser and a high sensitivity
142 digital camera system (OrcaFlash2.8, Hamamatsu, NanoSight Ltd) that determines the
143 Brownian motion of nanoparticles in real-time to assess size and concentration. Sixty-second
144 videos were recorded and particle movement was analysed using NTA software (version 2.3,
145 Fig 1B). Camera shutter speed was fixed at 30.01 ms. Camera gain was fixed to 500. Camera
146 sensitivity and detection threshold were (14-16) and (4-5), respectively. MP samples were
147 diluted in MP free - sterile water (Fresenius Kabi, Runcorn, UK). Samples were run in
148 quintuplicate, from which MP distribution, average concentration and mode size was
149 calculated.

150

151 **Flow cytometry**

152 Flow cytometric measurements were performed using a custom-built FACSAria II (BD
153 Biosciences, San Jose, CA, USA). Forward scatter area and side scatter area were set to log
154 scale. Data were exported from FACSDiva™ software version 6.0 (BD Biosciences) and
155 subsequently analysed with FlowJo software version 9.6.4 (Tree Star Inc, Ashland, OR,
156 USA). Plasma-derived MPs were resuspended in 100 µl of 0.22 µm-filtered annexin V
157 binding buffer (BD Biosciences). MPs were then stained for 15 min in the dark at room
158 temperature with annexin V-FITC (1.57 µg/ml), αCD41-PECy5 (0.12 µg/ml), αCD11b-
159 PECy7 (7.9 ug/ml) and αCD144-APC (4.1 µg/ml) (BioLegend, San Diego, CA, USA).
160 Fluorescent calibration beads of sizes 200, 500 and 800 nm were detected and distinguishable
161 as three distinct populations (Submicron bead calibration kit, Bangs Laboratories, Inc. IN,
162 USA). The MP gating strategy was based on their forward scatter versus side scatter profile
163 and in relation to platelets in fresh plasma. The MP gate was tested for annexin V positivity

164 and subsequently for monocyte (CD11b), platelet (CD41) and endothelial (CD144) antigens
165 to determine PS exposure and the cellular origin of MPs. FSC-A threshold was set to 1000 to
166 minimise recording of debris. Fluorescence minus one (FMO) stains were used to set the
167 positive gates for each antibody.

168

169 **Lipid extraction and fatty acid analysis**

170 Fatty acid profiles were analysed using gas chromatography (GC) with a flame ionisation
171 detector (FID) as described previously (Garaiova, *et al*., 2007). Briefly, lipids were extracted
172 using the method of Garbus, *et al*, 1963. Fatty acid methyl esters (FAME) were prepared by
173 incubation for 2 hr with H_2SO_4 : methanol : toluene (2.5 : 65 : 32.5, v/v/v) at 70 °C. A known
174 amount of C17:0 (margaric acid, Nu-Chek Prep. Inc, MN, USA) was added as an internal
175 standard. FAME were analysed by gas chromatography (GC) using a Clarus 500 gas
176 chromatograph (Perkin-Elmer 8500, CT, USA), fitted with a 30 m × 0.25 mm i.d., 0.25 μm
177 film thickness capillary column (Elite 225, Perkin Elmer). The column temperature was held
178 at 170 °C for 3 min then temperature-programmed to 220 °C at 4 °C / min. Nitrogen was the
179 carrier gas at a flow rate 2 ml / min. FAME were identified routinely by comparing retention
180 times of peaks with those of standards (Supelco 37 Component FAME Mix, Sigma-Aldrich,
181 UK).

182

183 **Analysis of microRNA (miR) expression**

184 MP miR expression was analysed on a small subset of PCOS patients (Mean ± SD n = 6, age:
185 33.8 ± 5 yrs, BMI: 28 ± 5 kg/m^2) and healthy controls (n= 6, age: 29.3 ± 5 yrs, 28 ± 6 BMI
186 kg/m^2). Total RNA was extracted from equal volumes of isolated MPs with TRIzol LS
187 Reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. miR
188 profile analysis was performed using Toray 3D-Gene™ DNA Chip microarrays (Toray

189 Industries Inc, Tokyo, Japan) according to the manufacturer's protocol. Briefly, total MP
190 RNA was labelled with a mercury LNA microRNA Array Power Labelling kit (Exiqon,
191 Vedbaek, Denmark). Labelled miRNAs were hybridised onto 3D-Gene miRNA oligo chips
192 containing more than 1,600 antisense probe spots (Toray Industries Inc). The annotation and
193 oligonucleotide sequences of the probes correspond to miRBase database version 16. The
194 chips were washed stringently, and fluorescent signals were scanned and analysed with a 3D-
195 Gene™ Scanner 3000 (Toray Industries Inc). Hybridised probe spots with signal intensity
196 greater than the mean intensity plus two standard deviations of the background signal were
197 considered valid. The background average was subtracted from the signal intensity, which
198 was then multiplied by the normalisation factor (25 divided by the median signal intensity of
199 all the subtracted background data) to generate the normalised data. Additionally, miR 4700-
200 5p was selected for validation by standard quantitative PCR (qPCR, PCOS patients (n = 12,
201 age: 30 ± 6 yrs, BMI: 30 ± 6 kg/m^2) and healthy controls (n= 9, age: 25 ± 2 yrs, BMI: 26 ± 6
202 kg/m^2)). MP RNA fraction (25 ng), isolated as described for the microarray, was converted
203 into miR 4700-5p cDNA (and RNU48 housekeeping control cDNA) using miR 4700-5p and
204 RNU48 probes (Life Tech) in a reverse transcriptase reaction. 7.5ng cDNA was used in each
205 PCR reaction following the manufacturer's instructions. miR 4700-5p MP levels were
206 expressed as fold changes compared to healthy volunteers.

207

208 **Statistics**

209 Data were analysed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA,
210 USA). D'Agostino's K-squared test was used to check data for normality. Analysis between
211 groups was performed using the independent *t*-test or the Mann-Whitney *U*-test for normally
212 or non-normally distributed data, respectively. Spearman's rank correlation coefficients were
213 used to explore the strength of the relationship between MP concentration and biochemical
214 parameters. The normalised microarray data were subjected to a Quantile-Quantile

215 normalisation, $\log^2$ transformed then analysed using an unpaired Student's *t*-test. Results are
216 expressed as mean ± SD unless indicated. A *p*-value <0.05 was considered statistically
217 significant. We based our sample size calculations on previous data, which demonstrated a
218 0.55 fold shift in mean circulating MP concentration in women with hyperandrogenic PCOS
219 compared to control subjects (Koiou *et al.,* 2011). Thus, to detect a similar shift in MP
220 concentration, with >90% power at the 5% α level, we sought to recruit a minimum of 15
221 subjects within each group.

222 **Results**

223 **Clinical and metabolic characteristics**

224 Table 1 summarises the metabolic and clinical characteristics of the PCOS and healthy
225 volunteer groups. As expected, PCOS subjects had higher testosterone, and insulin response
226 to glucose challenge, indicating reduced insulin sensitivity, although fasting insulin resistance
227 (HOMA-IR) did not differ. No significant differences were observed between groups with
228 respect to age, BMI, waist/hip circumference, lipid profile, hsCRP or glucose area under the
229 curve (AUC).

230

231 **Circulating MP concentration and size**

232 PCOS subjects had increased total circulating MP concentration compared to healthy
233 volunteers (Mean ± SD; 11. 5 ± 5 x10^{12}/ml versus 10.0 ± 4 x10^{12}/ml, respectively; p = 0.03;
234 Fig. 1A). In PCOS subjects, total MP concentration correlated significantly with HOMA-IR
235 (r=0.53, p=0.03). MP mode size was similar in both groups (Mean ± SD; 123 ± 7 nm versus
236 114 ± 4 nm, respectively; p = 0.18; Fig. 1C (top right)). To assess MP distribution, MP
237 concentrations were grouped in 50 nm bin sizes (Fig. 1C, large). PCOS subjects displayed a
238 significantly elevated concentration of small MPs (in the exosomal range, <150 nm in
239 diameter), compared to healthy volunteers: ([0 – 50 nm]: 4.27 ± 1.08 x10^{8}/ml versus 2.8 ±
240 1.48 x10^{8}/ml, respectively, p = 0.002; [51-100 nm]: 3.71 ± 1.08 x10^{9}/ml versus 2.52 ± 1.07
241 x10^{9}/ml, respectively, p = 0.002; [101-150 nm]: 4.71 ± 1.92 x10^{9}/ml versus 3.38 ± 0.9
242 x10^{9}/ml, respectively, p = 0.001).

243

244 **Cellular origin of circulating MPs**

245 MP cellular origin was determined by flow cytometry using monoclonal antibodies specific
246 for the lineage markers CD41 (platelet), CD144 (endothelium) and CD11b (monocyte). In

247 order to adhere to standard definitions, MPs were defined as annexin V^+ vesicles <1 μm in
248 diameter. A greater percentage of annexin V^+ MPs was detected in PCOS subjects compared
249 to healthy controls (Mean ± SD; 84 ± 18 % versus 74 ± 24 %, respectively; $p = 0.05$; Fig 1D).
250 Platelet-derived MPs occupied by far the greatest proportion of circulating MPs in both PCOS
251 subjects and healthy volunteers (Mean ± SD; 99 ± 0.9 % versus 99 ± 2.5 %, respectively; $p =$
252 0.27; Fig. 1E). Annexin V and CD144 and CD11b positive MPs (endothelial and monocyte-
253 derived MPs, respectively) were infrequent (Fig. 1E). A similar trend was observed in the
254 annexin V negative MP population. Platelet-derived MPs occupied the largest proportion of
255 circulating MPs in both PCOS subjects and healthy volunteers (94 ± 4 % versus 94 ± 9 %,
256 respectively; $p = 0.8$). Annexin V negative but CD144 and CD11b positive MPs were
257 infrequent.

258

259 **Fatty acid analysis**

260 Since an altered lipid metabolism may be a feature of PCOS, we explored if MPs similarly
261 exhibited an altered fatty acid profile. Using GC-FID, we found that the total fatty acid
262 concentration of MPs was similar in PCOS subjects and healthy volunteers (Median (25 – 75
263 % interquartile range); 7 (5 – 10) pg /10^6 MPs versus 8 (4 – 14) pg/10^6 MPs, respectively; $p =$
264 0.39; Fig. 2A). No differences in individual MP fatty acid composition were found between
265 PCOS patients and healthy controls (Fig. 2B).

266 To assess whether MP fatty acid composition was unique to MPs and not simply reflecting
267 plasma fatty acid distribution, we also undertook an analysis of plasma fatty acids. In an
268 analysis of all PCOS and healthy volunteer samples, MP fatty acid composition was found to
269 be different from the fatty acid composition of plasma, whereby 14 fatty acids were
270 differentially enriched ($p < 0.05$, Table S1). No differences were found between PCOS
271 patients and healthy volunteers with respect to total plasma fatty acid concentrations (426 ±

272 99 µg / 100 µl and 335 ± 51 µg / 100 µl, respectively, p = 0.65, Fig. 2C) but individually,
273 C18: 2n6 (linoleic acid) was elevated in PCOS subjects compared to healthy controls<0.01.

274

275 **MP miR expression**

276 Toray 3D-Gene™ chip analysis was employed to profile the miR content of circulating MPs
277 in a subpopulation of PCOS patients and healthy controls. In excess of 1,600 antisense
278 probes were plated onto the miR chip. All subjects analysed had a total miR count of >500.
279 Similar miR expression profiles were observed between groups for the most highly expressed
280 miRs. However, among the lowly expressed miRs, 16 were differentially expressed between
281 groups (table 2). qPCR was used to validate the differentially expressed miR 4700-5p.
282 Women with PCOS displayed a threefold-elevated expression of miR 4700-5p compared to
283 healthy volunteers, but this did not quite reach significance ($p = 0.1$).

284

285 **Discussion**

286 Our study shows that patients with PCOS have increased concentrations of circulating
287 annexin-V positive MPs compared with age- and BMI-matched healthy controls. We have
288 shown that these MPs are predominantly platelet-derived, and speculate that these alterations
289 may contribute to an increased cardiovascular risk. Our results are consistent with the
290 findings from two previous studies in which platelet-derived MPs were found to be elevated
291 in lean (Koiou *et al*., 2011) and overweight/obese (Koiou *et al*., 2013) hyperandrogenic
292 patients with PCOS. However, we extend these observations to characterise the fatty acid and
293 miR profile of circulating MPs, and show an association between MP concentration and
294 insulin resistance in our population.

295 We detected a similar proportion of MPs derived from platelets, monocytes and endothelial
296 cells in PCOS patients and healthy controls. In accordance with previous reports, we found
297 that PMPs occupied the greatest percentage of circulating MPs (Nieuwland *et al*., 2000). In
298 contrast, others have found higher percentages of endothelial- and monocyte-derived MPs in
299 healthy subjects (43% and 10.4%, respectively) (Shah *et al.,* 2008), which may reflect
300 different methodologies and pre-analytic protocols. Previous studies have shown that PMP
301 concentrations are elevated in lean and overweight/obese women with PCOS compared to
302 controls (Koiou *et al*., 2011 and Koiou *et al.,* 2013)). These studies used CD41-directed flow
303 cytometry to assess PMPs only, hence they were unable to compare MP cellular origin. Using
304 NTA we found that the increases in MP concentration in subjects with PCOS were largely
305 due to an increased concentration of MPs in the small (<150nm), exosomal range. This may
306 suggest selective stimulation of the intracellular classical exosomal pathway compared to
307 larger MPs (150-1000 nm diameter) formed via cell membrane shedding.

308 Koiou *et al.,* (2011) found a weak, but significant correlation between PMPs and serum
309 testosterone levels in their study of lean patients with PCOS. In contrast, we noted a
310 moderately strong correlation of MP concentration with HOMA-IR in PCOS subjects,

311 suggesting that elevated MP levels may be attributable, at least in part, to increased insulin
312 resistance. This is in line with several reports of increased MP concentrations in patients with
313 type 2 diabetes (Feng *et al.,* 2010, Koga *et al.,* 2005, Tramontano *et al*., 2010) including those
314 with end-organ damage (Omoto *et al.,* 1999). Metabolic syndrome, a disorder underpinned by
315 insulin insensitivity, is also characterised by an increased circulating MP concentration
316 compared to healthy controls (Arteaga *et al.,* 2006, Agouni *et al.,* 2008, Agouni *et al.,* 2011),
317 where they may contribute to endothelial dysfunction via increased oxidative stress (Agouni
318 *et al.,* 2011) and reduced nitric oxide synthase expression (Agouni *et al.,* 2008).
319 Hyperglycaemia (Terrisse *et al.,* 2010), inflammation and stress (Augustine *et al.,* 2014)
320 might also contribute to MP production. We also found a greater percentage of annexin V^+
321 MPs in PCOS patients. The extent of annexin V staining is largely taken to reflect binding to
322 phosphatidylserine which increases the potency for target cell interactions and may contribute
323 to enhanced pro-coagulant activity (Sinauridze *et al.,* 2007). We were unable to confirm any
324 differences in MP fatty acid composition between PCOS patients and healthy controls.
325 However, MP fatty acid composition was significantly different from that of plasma, perhaps
326 indicating that MPs are 'packaged' with a unique fatty acid signature rather than merely
327 reflecting the fatty acid composition of their environment.

328 To our knowledge, our study is the first to investigate the miR content of circulating MPs in
329 patients with PCOS. In an exploratory sub-population we found similar miR expression
330 profiles among women with PCOS and healthy volunteers for the most highly expressed
331 miRs. However, 16 lowly-expressed miRs were found to be differentially expressed. Of these,
332 miR-1293, miR-551a and miR-574-3p may be particularly noteworthy, as these target cellular
333 functions of relevance to PCOS pathology. miR-1293 targets peroxisome proliferator-
334 activated receptor gamma (PPAR-γ) co-activator (PPARGCA1), a pivotal regulator of
335 glucose homeostasis. miR-551a regulates hexose-6-phosphate dehydrogenase (H6PD),
336 mutations of which are recognised as a cause of hyperandrogenic PCOS (Martínez-García, *et*
337 *al.*, 2012), whilst mir-574-3p targets the follicle-stimulating hormone beta-subunit (FSHB)

and follicle-stimulating hormone receptor (FSHR) as previously noted in ovarian follicle fluid of PCOS patients (Sang *et al.,* 2013).

There are a number of potential limitations to our study. Firstly, we classified our PCOS patients by the Rotterdam criteria, which describes a less severe metabolic phenotype than other definitions of the syndrome (Carmina *et al.*, 2005). Our findings may thus not necessarily be generalisable to all patients with PCOS, but the presence of an altered MP profile in our young, mildly insulin resistant population suggests that changes in MP expression may occur early in the disease course. Secondly, miR expression analysis was only undertaken in an exploratory subset of the overall study population, hence validation of our findings in a larger cohort is mandatory. Furthermore, miR levels were unaltered for the highly expressed miRs and it is unclear whether differences in the lowly expressed miRs carries pathological relevance. Finally, methodological variability at both the sample preparation and analysis stage may make inter-study comparisons difficult. Whilst we sought to minimise the number of centrifugation steps, it is conceivable that platelet contamination might generate platelet-derived MPs in the freeze-thaw process. Additionally, whilst flow cytometry is acknowledged as the current gold standard for the determination of MP origin, the detection of smaller MPs (<400nm) is imperfect and it cannot observe the entire spectrum of MPs assessed using NTA.

In summary, our study suggests that patients with PCOS have an elevated concentration of circulating MPs compared with healthy controls. We show that these are predominantly platelet-derived, are associated with increased annexin V binding and an altered miR expression profile. Further studies are needed to confirm our findings, to explore the relevance of such changes to cardiovascular risk in women with PCOS, and to establish whether therapies that improve insulin sensitivity are able to reduce circulating MP concentrations.

Authors' roles

G.R.W. participated in study design and execution, data collection, analysis, and manuscript writing. K.C., K.L., T.S.D., I.A.G., D.R., K.M., D.A.P. and A.C. participated in study execution, data collection and analysis. P.E.J. and D.A.R. contributed to study design, manuscript writing and final approval.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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494 **Tables**

	Polycystic ovary syndrome (17)	**Healthy Controls (18)**	***p* value**
Age (years)	31 ± 6	31 ± 7	0.9
Weight (kg)	78 ± 21	76 ± 15	0.68
BMI (kg/m^2)	30 ± 6	29 ± 6	0.61
Waist (cm)	91 ± 15	86 ± 13	0.31
Hip (cm)	111 ± 16	106 ± 12	0.24
Testosterone (nmol/l)	1.4 ± 0.6	0.9 ± 0.6	0.02
hsCRP (mg/l)	1.25 (0.24 - 21.8)	0.9 (0.17 - 16.73)	0.73
Total cholesterol (mmol/l)	4.6 ± 1.3	4.8 ± 1.1	0.67
Triglycerides (mmol/l)	1.2 ± 1.4	1.0 ± 0.5	0.52
LDL cholesterol (mmol/l)	2.4 ± 1.4	2.5 ± 1.3	0.79
HDL cholesterol (mmol/l)	1.2 ± 0.5	1.3 ± 0.6	0.65
Insulin AUC (nmol min/l)	81 ± 46.7	53 ± 29.6	0.04
Glucose AUC (mmol min/l)	764 ± 217	692 ± 133	0.24
HOMA-IR	2 ± 0.9	2.5 ± 2.44	0.39

495

496 **Table 1. Demographic, anthropometric and metabolic characteristics of the study**
497 **population**. Data are presented as mean ± SD or median (range). hsCRP, high sensitivity C-
498 reactive protein; LDL, low density lipoprotein; HDL, high density lipoprotein; AUC, area
499 under the curve; HOMA-IR, homeostasis model assessment of insulin resistance.

500

microRNA	Expression fold change	*p* value
hsa-miR-551a	0.91	<0.001
hsa-miR-4324	0.80	0.007
hsa-miR-3689b, hsa-miR-3689c	1.11	0.009
hsa-miR-1293	0.84	0.012
hsa-miR-3936	1.10	0.012
hsa-miR-4481	0.88	0.019
hsa-miR-629	1.16	0.019
hsa-miR-4425	1.19	0.019
hsa-miR-30b	0.89	0.021
hsa-miR-3622a-3p	0.86	0.022
hsa-miR-514b-5p	0.83	0.025
hsa-miR-4700-5p	1.25	0.029
hsa-miR-4708-3p	0.88	0.037
hsa-miR-574-3p	1.19	0.038
hsa-miR-4283	0.85	0.041
hsa-miR-23a	0.86	0.043
hsa-miR-3156-5p	1.18	0.047

501

502 **Table 2. Differentially expressed microRNAs (miRs) in circulating microparticles.** Fold
503 change was calculated as average polycystic ovary syndrome miR expression / average
504 healthy control miR expression. All samples tested had total miR counts >500.

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	Microparticle (MP)		Plasma		
Fatty Acid	**Mean**	**SD**	**Mean**	**SD**	***p* value**
C14 :0*	0.93%	0.60%	0.67%	0.35%	0.038
C14: 1*	0.20%	0.21%	0.09%	0.11%	0.012
C16: 0*	27.32%	5.82%	20.80%	3.27%	<0.001
C16: 1	0.56%	0.64%	0.78%	1.94%	0.519
C16: 1n7	1.59%	2.15%	1.82%	0.68%	0.562
C18: 0*	12.95%	4.07%	7.59%	1.24%	<0.001
C18: 1n9*	30.69%	5.58%	22.07%	3.32%	<0.001
C18 1n7*	0.36%	1.73%	1.67%	2.94%	0.028
C18 2n6*	12.72%	6.04%	28.20%	4.05%	<0.001
C18 3n6*	0.20%	0.20%	0.42%	0.20%	<0.001
C18: 3n3*	0.51%	0.43%	0.74%	0.24%	0.008
C20: 0	0.56%	0.53%	0.93%	2.91%	0.466
C20: 1	0.06%	0.16%	0.00%	0.00%	0.058
C20: 2n6*	0.95%	0.61%	1.67%	0.41%	<0.001
C20: 4n6	5.74%	3.85%	6.83%	1.61%	0.139
C20: 5n3*	0.31%	0.25%	0.77%	0.30%	<0.001
C22:0*	1.40%	0.95%	0.48%	0.20%	<0.001
C22: 3n3	0.39%	0.55%	0.23%	0.27%	0.137
C22: 3n6	0.23%	0.25%	0.25%	0.13%	0.743
C22: 5n3	0.34%	0.32%	0.44%	0.13%	0.114
C22: 6n3*	0.86%	0.68%	1.83%	0.54%	<0.001
C24: 0	0.51%	1.06%	0.33%	0.13%	0.346
C24: 1n9*	0.33%	0.27%	0.70%	0.27%	<0.001

512

513 **Table S1. Comparison between microparticle (MP) and plasma fatty acid composition**

514 **(% of total peak area).** Mean and standard deviation (SD) values represent data from across

515 both subject groups. * denotes significance.

516

517 **Legends for figures**

518 **Fig 1. Quantification of circulating microparticles (MPs).** (**A**) Plasma MP concentration
519 in 17 polycystic ovary syndrome (PCOS) patients and 18 healthy controls determined by
520 nanoparticle tracking analysis (NTA). (**B**) Representative image showing determination of
521 MP size and concentration by Brownian motion of plasma MPs (NTA analysis software
522 version 2.3). (**C**) Plasma MP distribution; presented in 50 nm bin sizes (larger figure) and
523 mode MP size (smaller figure). (**D**) The percentage of annexin V positive MPs in PCOS
524 patients and healthy controls determined by flow cytometric analysis. (**E**) Plasma MP cell
525 origin determined by flow cytometric analysis of the lineage-specific markers CD41
526 (platelet), CD144 (endothelium) and CD11b (monocyte) on annexin V^+ vesicles <1 μm in
527 diameter. Data are presented as mean ± SEM. * denotes $p < 0.05$.

528

529 **Fig 2. Fatty acid analysis.** Gas chromatography coupled with a flame ionisation detector was
530 used to measure fatty acids in plasma and circulating MPs. (**A**) Total fatty acid (FA)
531 concentrations in circulating MPs. (**B**) Individual FA composition of circulating MPs. (**C**)
532 Total FA concentrations in plasma. (**D**) Individual FA composition of plasma. Data are
533 presented as mean ± SEM. * denotes $p < 0.05$.

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