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

G.R. Willis¹, K. Connolly¹, K. Ladell², T.S. Davies³, I.A. Guschina³, D. Ramji³, K. Miners², D.A. Price², A. Clayton⁴, P.E. James¹, and D.A. Rees¹,*

¹Institute of Molecular and Experimental Medicine, Cardiff University, Cardiff CF14 4XN, UK ²Institute of Infection and Immunity, Cardiff University, Cardiff CF14 4XN, UK ³School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK ⁴Institute of Cancer and Genetics, Cardiff University, Velindre Cancer Centre, Whitchurch, Cardiff CF14 2TL, UK

*Correspondence address. Tel: +44-2920-742341; Fax: +442920-744671; E-mail: reesda@cf.ac.uk

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STUDY QUESTION: Are circulating microparticles (MPs) altered in young women with polycystic ovary syndrome (PCOS)?

SUMMARY ANSWER: Women with PCOS have elevated concentrations of circulating platelet-derived MPs, which exhibit increased annexin V binding and altered microRNA (miR) profiles compared with healthy volunteers.

WHAT IS KNOWN ALREADY: Some studies have shown that cardiovascular risk is increased in young women with PCOS but the mechanisms by which this occurs are uncertain. Circulating MPs are elevated in patients with cardiovascular disease but the characteristics of MPs in patients with PCOS are unclear.

STUDY DESIGN, SIZE, DURATION: Case–control study comprising 17 women with PCOS (mean ± SD; age 31 ± 7 years, BMI 29 ± 6 kg/m²) and 18 healthy volunteers (age 31 ± 6 years, BMI 30 ± 6 kg/m²).

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study was conducted in a University hospital. Nanoparticle tracking analysis (NTA) and flow cytometry (CD41 platelet, CD11b monocyte, CD144 endothelial) were used to determine MP size, concentration, cellular origin and annexin V positivity (reflecting phosphatidylserine exposure). Fatty acid analysis was performed by gas chromatography and MP miR expression profiles were compared by microarray.

MAIN RESULTS AND THE ROLE OF CHANCE: PCOS subjects showed increased MP concentrations compared with healthy volunteers (mean ± SD; 11.5 ± 5 × 10¹²/ml versus 10.0 ± 4 × 10¹²/ml, respectively; P = 0.03), which correlated with the homeostasis model of insulin resistance (r = 0.53, P = 0.03). This difference was predominantly seen in MPs whose size was in the small exosomal range (<150 nm in diameter, P < 0.05). PCOS patients showed a greater percentage of annexin V+ MPs compared with healthy volunteers (84 ± 18 versus 74 ± 24%, respectively, P = 0.05) but the cellular origin of MPs, which were predominantly platelet-derived (PCOS: 99 ± 0.9%; controls: 99 ± 2.5%), did not differ. MP fatty acid concentration and composition was similar between groups but 16 miRs were differentially expressed (P < 0.05).

LIMITATIONS, REASON FOR CAUTION: Patients with PCOS were classified by the Rotterdam criteria, which describes a less severe metabolic phenotype than other definitions of the syndrome. Our findings may thus not be generalizable to all patients with PCOS. MicroRNA 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.

WIDER IMPLICATIONS OF THE FINDINGS: This study suggests that women with PCOS have an altered MP profile but further studies are needed to confirm this, to explore the mechanisms by which these alterations develop and to establish whether therapies that improve insulin sensitivity are able to reduce circulating MP concentrations.

STUDY FUNDING/COMPETING INTEREST(S): The study was funded by grants from the Wales Heart Research Institute and Mrs John
Introduction

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

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

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

Methods

Subjects and protocol

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

Blood sampling, isolation and storage of microparticles

Fasting blood samples were drawn from an antecubital vein into ethylene-diaminetetraacetic acid vacutainers. Blood samples were promptly centrifuged (1024 g × 10 min at 4 °C) to yield platelet-poor plasma. Plasma-derived MPs were isolated via differential ultracentrifugation. Briefly, plasma (1 ml) was ultracentrifuged (100 000 × g × 1 h at 4 °C; Beckman Coulter, UK) and the supernatant was discarded, as previously described (Connolly et al., 2014). The remaining pellet was resuspended in 250 μl of RNAase-free phosphate-buffered saline (Fisher Scientific, UK) which had been filtered using a 0.22 μm Millipore (Merck Millipore, UK). Isolated MPs were stored at −80 °C, for no longer than 6 months before analysis. For use, samples were thawed in a pre-heated (37 °C) thermostatically-regulated water bath for 3 min.

Nanoparticle tracking analysis

MP size and concentration were determined using NTA (NanoSight LM10 system, UK) as described previously (Webber and Clayton, 2013). Briefly, NTA is a laser illuminated microscopic technique equipped with a 405 nm laser and a high sensitivity digital camera system (OrcaFlash2.8, Hamamatsu, NanoSight Ltd) that determines the Brownian motion of nanoparticles in real-time to assess size and concentration. Sixty-second videos were recorded and particle movement was analysed using NTA software (version 2.3, Fig. 1B). Camera shutter speed was fixed at 30.01 ms. Camera gain was fixed to 500. Camera sensitivity and detection threshold were (14–16) and (4–5), respectively. MP samples were diluted in MP-free sterile water (Fresenius Kabi, Runcorn, UK). Samples were run in
Figure 1  Quantification of circulating microparticles (MPs). (A) Plasma MP concentration in 17 polycystic ovary syndrome (PCOS) patients and 18 healthy controls determined by nanoparticle tracking analysis (NTA). (B) Representative image showing determination of MP size and concentration by Brownian motion of plasma MPs (NTA analysis software version 2.3). (C) Plasma MP distribution; presented in 50 nm bin sizes (larger figure) and mode MP size (smaller figure). (D) The percentage of annexin V positive MPs in PCOS patients and healthy controls determined by flow cytometric analysis. (E) Plasma MP cell origin determined by flow cytometric analysis of the lineage-specific markers CD41 (platelet), CD144 (endothelium) and CD11b (monocyte) on annexin V⁺ vesicles < 1 μm in diameter. Data are presented as mean ± SEM. * denotes P < 0.05.
quintuplicate, from which MP distribution, average concentration and mode size were calculated.

**Flow cytometry**

Flow cytometric measurements were performed using a custom-built FACSAna II (BD Biosciences, San Jose, CA, USA). Forward scatter area and side scatter area were set to log scale. Data were exported from FACSData software version 6.0 (BD Biosciences) and subsequently analysed with FlowJo software version 9.6.4 (Tree Star, Inc., Ashland, OR, USA). Plasma-derived MPs were resuspended in 100 µl of 0.2% 2-µm-filtered annexin V binding buffer (BD Biosciences). MPs were then stained for 15 min in the dark at room temperature with annexin V-FITC (1.57 µg/ml), αCD41-PECy5 (0.12 µg/ml), αCD11b-PECy7 (7.9 µg/ml) and αCD144-APC (4.1 µg/ml) (BioLegend, San Diego, CA, USA). Fluorescent calibration beads of sizes 200, 500 and 800 nm were detected and distinguishable as three distinct populations (Submicron bead calibration kit, Bangs Laboratories, Inc., IN, USA). The MP gating strategy was based on their forward scatter versus side scatter profile and in relation to platelets in fresh plasma. The MP gate was tested for annexin V positivity and subsequently for monocyte (CD11b), platelet (CD41) and endothelial (CD144) antigens to determine PS exposure and the cellular origin of MPs. FSC-A threshold was set to 1000 to minimize recording of debris. Fluorescence minus one (PMO) stains were used to set the positive gates for each antibody.

**Lipid extraction and fatty acid analysis**

Fatty acid profiles were analysed using gas chromatography (GC) with a flame ionization detector (FID) as described previously (Garbus et al., 1963). Briefly, lipids were extracted using the method of Garbus et al. (1963). Fatty acid methyl esters (FAME) were prepared by incubation for 2 h with H₂SO₄: methanol: toluene (2.5: 65: 32.5, v/v/v) at 70°C. A known amount of C17:0 (margaric acid, Nu-Chek Prep. Inc., MN, USA) was added as an internal standard. FAME were analysed by gas chromatography (GC) using a Clarus 500 gas chromatograph (Perkin-Elmer 8500, CT, USA), fitted with a 30 m × 0.25 mm i.d., 0.25 µm film thickness capillary column (Elite 225, Perkin Elmer). The column temperature was held at 170°C for 3 min then temperature-programmed to 220°C at 4°C/min. Nitrogen was the carrier gas at a flow rate 2 ml/min. FAME were identified routinely by comparing retention times of peaks with those of standards (Supelco 37 Component FAME Mix, Sigma-Aldrich, UK).

**Analysis of microRNA (miR) expression**

MP miR expression was analysed on a small subset of PCOS patients (mean ± SD n = 6, age: 33.8 ± 5 years, BMI: 28 ± 5 kg/m²) and healthy controls (n = 6, age: 29.3 ± 5 years, 28 ± 6 BMI kg/m²). Total RNA was extracted from equal volumes of isolated MPs with TRIzol LS Reagent (Ambion, Austin, TX, USA). Plasma-derived MPs were resuspended in 100 µl of 0.2% 2-µm-filtered annexin V binding buffer (BD Biosciences). MPs were then stained for 15 min in the dark at room temperature with annexin V-FITC (1.57 µg/ml), αCD41-PECy5 (0.12 µg/ml), αCD11b-PECy7 (7.9 µg/ml) and αCD144-APC (4.1 µg/ml) (BioLegend, San Diego, CA, USA). Fluorescent calibration beads of sizes 200, 500 and 800 nm were detected and distinguishable as three distinct populations (Submicron bead calibration kit, Bangs Laboratories, Inc., IN, USA). The MP gating strategy was based on their forward scatter versus side scatter profile and in relation to platelets in fresh plasma. The MP gate was tested for annexin V positivity and subsequently for monocyte (CD11b), platelet (CD41) and endothelial (CD144) antigens to determine PS exposure and the cellular origin of MPs. FSC-A threshold was set to 1000 to minimize recording of debris. Fluorescence minus one (PMO) stains were used to set the positive gates for each antibody.

by standard quantitative PCR (qPCR). PCOS patients (n = 12, age: 30 ± 6 years, BMI: 30 ± 6 kg/m²) and healthy controls (n = 9, age: 25 ± 2 years, BMI: 26 ± 6 kg/m²) were compared with healthy volunteers. (RNU48 house-keeping control cDNA) using miR 4700-5p and RNU48 probes (Life Tech) in a reverse transcriptase reaction. 7.5 ng cDNA was used in each qPCR reaction following the manufacturer’s instructions. miR 4700-5p MP levels were expressed as fold changes compared with healthy volunteers.

**Statistics**

Data were analysed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). D’Agostino’s K-squared test was used to check for normality. Analysis between groups was performed using the independent t-test or the Mann-Whitney U-test for normally or non-normally distributed data, respectively. Spearman’s rank correlation coefficients were used to explore the strength of the relationship between MP concentration and biochemical parameters. The normalized microarray data were subjected to a quantile-quantile normalization, log₅ transformed and then analysed using an unpaired Student’s t-test. Results are expressed as mean ± SD unless indicated. A P-value < 0.05 was considered statistically significant. We based our sample size calculations on previous data, which demonstrated a 0.55-fold shift in mean circulating MP concentration in women with hyperandrogenic PCOS compared with control subjects (Koiou et al., 2011). Thus, to detect a similar shift in MP concentration, with >90% power at the 5% α level, we sought to recruit a minimum of 15 subjects within each group.

**Results**

**Clinical and metabolic characteristics**

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

**Circulating MP concentration and size**

PCOS subjects had increased total circulating MP concentration compared with healthy volunteers (mean ± SD: 11.5 ± 5 × 10¹²/ml versus 10.0 ± 4 × 10¹²/ml, respectively; P = 0.03; Fig. 1A). In PCOS subjects, total MP concentration correlated significantly with HOMA-IR (r = 0.53, P = 0.03). MP mode size was similar in both groups (mean ± SD: 123 ± 7 versus 114 ± 4 nm, respectively; P = 0.18; Fig. 1C (top right)). To assess MP distribution, MP concentrations were grouped in 50 nm bin sizes (Fig. 1C, large). PCOS subjects displayed a significantly elevated concentration of small MPs (in the exosomal range, <150 nm in diameter), compared with healthy volunteers: ([0–50 nm]: 4.27 ± 1.08 × 10⁶/ml versus 2.8 ± 1.48 × 10⁶/ml, respectively, P = 0.002; [51–100 nm]: 3.71 ± 1.08 × 10⁷/ml versus 2.52 ± 1.07 × 10⁷/ml, respectively, P = 0.002; [101–150 nm]: 4.71 ± 1.92 × 10⁸/ml versus 3.38 ± 0.9 × 10⁸/ml, respectively, P = 0.001).

**Cellular origin of circulating MPs**

MP cellular origin was determined by flow cytometry using monoclonal antibodies specific for the lineage markers CD41 (platelet), CD144...
acids were differentially enriched (P different from the fatty acid composition of plasma, whereby 14 fatty healthy volunteer samples, MP fatty acid composition was found to be took an analysis of plasma fatty acids. In an analysis of all PCOS and and not simply reflecting plasma fatty acid distribution, we also under-

Fig. 2C). Analysis of individual fatty acids showed that the differences in individual MP fatty acid composition were found between PCOS patients and healthy controls (Fig. 2B).

Since an altered lipid metabolism may be a feature of PCOS, we explored

| Table I Demographic, anthropometric and metabolic characteristics of the study population. |
|---------------------------------|---------------------------------|----------------|
| Age (years)                     | 31 ± 6                          | 31 ± 7         |
| Weight (kg)                     | 78 ± 21                         | 76 ± 15        |
| BMI (kg/m²)                     | 30 ± 6                          | 29 ± 6         |
| Waist (cm)                      | 91 ± 15                         | 86 ± 13        |
| Hip (cm)                        | 111 ± 16                        | 106 ± 12       |
| Testosterone (nmol/l)           | 1.4 ± 0.6                       | 0.9 ± 0.6      |
| hsCRP (mg/l)                    | 1.25 (0.24–21.8)                | 0.9 (0.17–16.73)|
| Total cholesterol (mmol/l)      | 4.6 ± 1.3                       | 4.8 ± 1.1      |
| Triglycerides (mmol/l)          | 1.2 ± 1.4                       | 1.0 ± 0.5      |
| LDL cholesterol (mmol/l)        | 2.4 ± 1.4                       | 2.5 ± 1.3      |
| HDL cholesterol (mmol/l)        | 1.2 ± 0.5                       | 1.3 ± 0.6      |
| Insulin AUC (nmol min/l)        | 81 ± 46.7                       | 53 ± 29.6      |
| Glucose AUC (mmol min/l)        | 764 ± 217                       | 692 ± 133      |
| HOMA-IR                         | 2 ± 0.9                         | 2.5 ± 2.44     |

Data are presented as mean ± SD or median (range).

hsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AUC, area under the curve; HOMA-IR, homeostasis model assessment of insulin resistance.

(endothelium) and CD11b (monocyte). In order to adhere to standard definitions, MPs were defined as annexin V+ vesicles < 1 μm in diameter. A greater percentage of annexin V+ MPs was detected in PCOS subjects compared with healthy controls (mean ± SD; 84 ± 18 versus 74 ± 24%, respectively; P = 0.05; Fig. 1D). Platelet-derived MPs occupied by far the greatest proportion of circulating MPs in both PCOS subjects and healthy volunteers (mean ± SD; 99 ± 9 versus 99 ± 2.5%, respectively; P = 0.27; Fig. 1E). Annexin V and CD144 and CD11b positive MPs (endothelial and monocyte-derived MPs, respectively) were in-frequent (Fig. 1E). A similar trend was observed in the annexin V negative MP population. Platelet-derived MPs occupied the largest proportion of circulating MPs in both PCOS subjects and healthy volunteers (94 ± 4 versus 94 ± 9%, respectively; P = 0.8). Annexin V negative but CD144 and CD11b positive MPs were infrequent.

**Fatty acid analysis**

Since an altered lipid metabolism may be a feature of PCOS, we explored if MPs similarly exhibited an altered fatty acid profile. Using GC-FID, we found that the total fatty acid concentration of MPs was similar in PCOS subjects and healthy volunteers (median (quartiles) 7 (5–10) pg/10⁶ MPs versus 8 (4–14) pg/10⁶ MPs, respectively; P = 0.39; Fig. 2A). No differences in individual MP fatty acid composition were found between PCOS patients and healthy controls (Fig. 2B).

To assess whether MP fatty acid composition was unique to MPs and not simply reflecting plasma fatty acid distribution, we also undertook an analysis of plasma fatty acids. In an analysis of all PCOS and healthy volunteer samples, MP fatty acid composition was found to be different from the fatty acid composition of plasma, whereby 14 fatty acids were differentially enriched (P < 0.05, Supplementary data, Table SI). No differences were found between PCOS patients and healthy volunteers with respect to total plasma fatty acid concentrations (426 ± 99 μg/100 μl and 335 ± 51 μg/100 μl, respectively, P = 0.65, Fig. 2C). Analysis of individual fatty acids showed that the concentration of several plasma fatty acids was elevated in PCOS subjects including C14: 1 (myristoleic acid), C16: 1n9 (hexadecenoic acid), C22: 3n6 (docosatrienoic acid) and C22: 5n3 (docosapentaenoic acid) (all P < 0.05).

**MP miR expression**

Toray 3D-Gene™ chip analysis was employed to profile the miR content of circulating MPs in a subpopulation of PCOS patients and healthy controls. In excess of 1600 antisense probes were plated onto the miR chip. All subjects analysed had a total miR count of >500. Similar miR expression profiles were observed between groups for the most highly expressed miRs. However, among the lowly expressed miRs, 16 were differentially expressed between groups (Table II). qPCR was used to validate the differentially expressed miR 4700-5p. Women with PCOS displayed a 3-fold elevated expression of miR 4700-5p compared with healthy volunteers, but this did not quite reach significance (P = 0.1).

**Discussion**

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

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

Koiou et al. (2011) found a weak, but significant correlation between PMPs and serum testosterone levels in their study of lean patients with PCOS. In contrast, we noted a moderately strong correlation of MP concentration with HOMA-IR in PCOS subjects, suggesting that elevated MP levels may be attributable, at least in part, to increased insulin resistance. This is in line with several reports of increased MP concentrations in patients with type 2 diabetes (Koga et al., 2005; Feng et al., 2010; Tramontano et al., 2010) including those with end-organ damage (Omoto et al., 1999). Metabolic syndrome, a disorder underpinned by insulin insensitivity, is also characterized by an increased circulating MP concentration compared with healthy controls (Arteaga et al., 2006; Agouni et al., 2008, 2011), where they may contribute to endothelial dysfunction via increased oxidative stress (Agouni et al., 2011) and reduced nitric oxide synthase expression (Agouni et al., 2008). Hyperglycaemia

### Table II Differentially expressed microRNAs (miRs) in circulating microparticles.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Expression fold change</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-551a</td>
<td>0.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsa-miR-4324</td>
<td>0.80</td>
<td>0.007</td>
</tr>
<tr>
<td>hsa-miR-3689b</td>
<td>1.11</td>
<td>0.009</td>
</tr>
<tr>
<td>hsa-miR-3689c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsa-miR-1293</td>
<td>0.84</td>
<td>0.012</td>
</tr>
<tr>
<td>hsa-miR-3936</td>
<td>1.10</td>
<td>0.012</td>
</tr>
<tr>
<td>hsa-miR-4481</td>
<td>0.88</td>
<td>0.019</td>
</tr>
<tr>
<td>hsa-miR-629</td>
<td>1.16</td>
<td>0.019</td>
</tr>
<tr>
<td>hsa-miR-4425</td>
<td>1.19</td>
<td>0.019</td>
</tr>
<tr>
<td>hsa-miR-30b</td>
<td>0.89</td>
<td>0.021</td>
</tr>
<tr>
<td>hsa-miR-3622a-3p</td>
<td>0.86</td>
<td>0.022</td>
</tr>
<tr>
<td>hsa-miR-514b-5p</td>
<td>0.83</td>
<td>0.025</td>
</tr>
<tr>
<td>hsa-miR-4700-5p</td>
<td>1.25</td>
<td>0.029</td>
</tr>
<tr>
<td>hsa-miR-4708-3p</td>
<td>0.88</td>
<td>0.037</td>
</tr>
<tr>
<td>hsa-miR-574-3p</td>
<td>1.19</td>
<td>0.038</td>
</tr>
<tr>
<td>hsa-miR-4283</td>
<td>0.85</td>
<td>0.041</td>
</tr>
<tr>
<td>hsa-miR-23a</td>
<td>0.86</td>
<td>0.043</td>
</tr>
<tr>
<td>hsa-miR-3156-5p</td>
<td>1.18</td>
<td>0.047</td>
</tr>
</tbody>
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Fold change was calculated as average polycystic ovary syndrome miR expression/average healthy control miR expression. All samples tested had total miR counts >500.
(Terrisse et al., 2010), inflammation and stress (Augustine et al., 2014) might also contribute to MP production. We also found a greater percentage of annexin V+ MPs in PCOS patients. The extent of annexin V staining is largely taken to reflect binding to phosphatidylserine which increases the potency for target cell interactions and may contribute to enhanced pro-coagulant activity (Snauridze et al., 2007). We were unable to confirm any differences in MP fatty acid composition between PCOS patients and healthy controls. However, MP fatty acid composition was significantly different from that of plasma, perhaps indicating that MPs are ‘packaged’ with a unique fatty acid signature rather than merely reflecting the fatty acid composition of their environment.

To our knowledge, our study is the first to investigate the miR content of circulating MPs in patients with PCOS. In an exploratory subpopulation we found similar miR expression profiles among women with PCOS and healthy volunteers for the most highly expressed miRs. However, 16 lowly expressed miRs were found to be differentially expressed. Of these, miR-1293, miR-551a and miR-574-3p may be particularly noteworthy, as these target cellular functions of relevance to PCOS pathology. miR-1293 targets peroxisome proliferator-activated receptor gamma (PPAR-γ) co-activator (PPARGCA1), a pivotal regulator of glucose homeostasis. miR-551a regulates hexose-6-phosphate dehydrogenase (H6PD), mutations of which are recognized as a cause of hyperandrogenic PCOS (Martinez-Garcia et 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 generalizable 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 minimize 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 (<400 nm) 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, and 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.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles

Funding
Our research was supported by grants from The Wales Heart Research Institute and Mrs John Nixon Scholarship.

Conflict of interest
None declared.

References
Garcìa et al., 2012, whilst mir-574-3p targets the follicle-stimulating hormone gamma (FSBHG) and follicle-stimulating hormone receptor (FSHR) as previously noted in ovarian follicle fluid of PCOS patients (Sang et al., 2013).

... is largely taken to reflect binding to phosphatidylserine which increases the potency for target cell interactions and may contribute to enhanced pro-coagulant activity (Snauridze et al., 2007). We were unable to confirm any differences in MP fatty acid composition between PCOS patients and healthy controls. However, MP fatty acid composition was significantly different from that of plasma, perhaps indicating that MPs are ‘packaged’ with a unique fatty acid signature rather than merely reflecting the fatty acid composition of their environment.

To our knowledge, our study is the first to investigate the miR content of circulating MPs in patients with PCOS. In an exploratory subpopulation we found similar miR expression profiles among women with PCOS and healthy volunteers for the most highly expressed miRs. However, 16 lowly expressed miRs were found to be differentially expressed. Of these, miR-1293, miR-551a and miR-574-3p may be particularly noteworthy, as these target cellular functions of relevance to PCOS pathology. miR-1293 targets peroxisome proliferator-activated receptor gamma (PPAR-γ) co-activator (PPARGCA1), a pivotal regulator of glucose homeostasis. miR-551a regulates hexose-6-phosphate dehydrogenase (H6PD), mutations of which are recognized as a cause of hyperandrogenic PCOS (Martinez-Garcia et 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 generalizable 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 minimize 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 (<400 nm) 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, and 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.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles

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Conflict of interest
None declared.

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Blood microparticles and polycystic ovary syndrome


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