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. Miners³, D.A. Price², A. Clayton⁴, P.E. James¹, 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.

Running title: Blood microparticles and polycystic ovary syndrome

*Correspondence address: Tel: +442920 742341; Fax: +442920 744671; email: reesda@cf.ac.uk
Abstract

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 to 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 is uncertain. Circulating MPs are elevated in patients with cardiovascular disease but the characteristics of MPs in patients with PCOS are unclear.

Study design: Case-control study comprising 17 women with PCOS (Mean ± SD; age 31 ± 7 yrs, BMI 29 ± 6 kg/m²) and 18 healthy volunteers (age 31 ± 6 yrs, BMI 30 ± 6 kg/m²).

Participants/materials, setting, methods: The study was conducted in a University hospital. Nanoparticle tracking analysis 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 to healthy volunteers (Mean ± SD; 11.5 ± 5 x10¹²/ml versus 10.0 ± 4 x10¹²/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 to 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 generalisable 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 Nixon Scholarship. The authors have no conflicts of interest to declare.

**Key words:** Polycystic Ovary Syndrome, microparticles, insulin resistance.
Diamond classification of diamond cut, shape, color and clarity. These factors determine the overall value and beauty of a diamond. 

Polycystic ovary syndrome (PCOS) is a common endocrine condition characterised by hyperandrogenism, polycystic ovaries and oligo/anovulation. In addition to its reproductive sequelae, PCOS is now considered a metabolic disorder characterised 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 (El-Kannishy et al., 2010, Orio et al., 2004), 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) (Koga et al., 2005, Mallat et al., 2000).

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. Koioiu 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 characterisation 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, Cushing’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 antidiabetics, 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.

Anthropometric and biochemical measurements

Subjects attended our Clinical Research Facility at 0800h after an overnight fast. Studies were conducted in a quiet, temperature-controlled room and subjects were required to rest for 10 minutes before study measurements. Height, weight, hip and waist circumference were measured as per our published protocols (Watson et al., 2009). Serum total cholesterol (TC), high density lipoprotein cholesterol (HDL), and triglycerides (TG) were assayed using an Aeraset automated analyser (Abbott Diagnostics, Berkshire, UK); low density lipoprotein
cholesterol (LDL) was calculated using Friedewald’s formula. Insulin was measured using an immunometric assay specific for human insulin (Invitron, Monmouth, UK) and glucose was measured using the Aeroset chemistry system (Abbott Diagnostics, Berkshire, UK). High sensitivity C-reactive protein (hsCRP) was assayed by nephelometry (BN™ II system; Dade-Behring, Milton Keynes, UK) and total testosterone was measured by liquid chromatography-tandem mass spectrometry (Quattro™ Premier XE triple quadrupole tandem mass spectrometer; Waters Ltd, Watford, UK). The intra- and inter-assay coefficients of variation were all less than 9%. After basal sampling, subjects underwent a standard 75g oral glucose tolerance test (OGTT). Glucose and insulin were measured at 0, 30, 60, 90 and 120 minutes. The area under the curve (AUC) for insulin and glucose was calculated using the trapezoid method. Fasting insulin resistance was also estimated by the homeostasis model assessment method (HOMA-IR).

Blood sampling, isolation and storage of microparticles

Fasting blood samples were drawn from an antecubital vein into ethylenediaminetetraacetic acid vacutainers. Blood samples were promptly centrifuged (1,024 g x 10min 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 x 1 hr 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 preheated (37 °C) thermostatically-regulated water bath for 3 minutes.
Nanoparticle tracking analysis (NTA)

MP size and concentration were determined using nanoparticle tracking analysis (NTA) (NanoSight LM10 system, UK) as described previously (Webber, 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 quintuplicate, from which MP distribution, average concentration and mode size was calculated.

Flow cytometry

Flow cytometric measurements were performed using a custom-built FACSAria II (BD Biosciences, San Jose, CA, USA). Forward scatter area and side scatter area were set to log scale. Data were exported from FACSDiva™ 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 0.22 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 ug/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
minimise recording of debris. Fluorescence minus one (FMO) 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 ionisation
detector (FID) as described previously (Garaiova, et al., 2007). Briefly, lipids were extracted
using the method of Garbus, et al., 1963. Fatty acid methyl esters (FAME) were prepared by
incubation for 2 hr 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 yrs, BMI: 28 ± 5 kg/m²) and healthy controls (n= 6, age: 29.3 ± 5 yrs, 28 ± 6 BMI
kg/m²). Total RNA was extracted from equal volumes of isolated MPs with TRIzol LS
Reagent (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. miR
profile analysis was performed using Toray 3D-Gene™ DNA Chip microarrays (Toray
Industries Inc, Tokyo, Japan) according to the manufacturer’s protocol. Briefly, total MP RNA was labelled with a mercury LNA microRNA Array Power Labelling kit (Exiqon, Vedbaek, Denmark). Labelled miRNAs were hybridised onto 3D-Gene miRNA oligo chips containing more than 1,600 antisense probe spots (Toray Industries Inc). The annotation and oligonucleotide sequences of the probes correspond to miRBase database version 16. The chips were washed stringently, and fluorescent signals were scanned and analysed with a 3D-Gene™ Scanner 3000 (Toray Industries Inc). Hybridised probe spots with signal intensity greater than the mean intensity plus two standard deviations of the background signal were considered valid. The background average was subtracted from the signal intensity, which was then multiplied by the normalisation factor (25 divided by the median signal intensity of all the subtracted background data) to generate the normalised data. Additionally, miR 4700-5p was selected for validation by standard quantitative PCR (qPCR, PCOS patients (n = 12, age: 30 ± 6 yrs, BMI: 30 ± 6 kg/m²) and healthy controls (n= 9, age: 25 ± 2 yrs, BMI: 26 ± 6 kg/m²)). MP RNA fraction (25 ng), isolated as described for the microarray, was converted into miR 4700-5p cDNA (and RNU48 housekeeping control cDNA) using miR 4700-5p and RNU48 probes (Life Tech) in a reverse transcriptase reaction. 7.5ng cDNA was used in each PCR reaction following the manufacturer’s instructions. miR 4700-5p MP levels were expressed as fold changes compared to 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 data 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 normalised microarray data were subjected to a Quantile-Quantile
normalisation, log² transformed 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 to 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 1 summarises 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 to healthy volunteers (Mean ± SD; 11.5 ± 5 x10^{12}/ml versus 10.0 ± 4 x10^{12}/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 nm 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 to healthy volunteers: (\([0 \text{–} 50 \text{ nm}]: 4.27 ± 1.08 \times 10^9/\text{ml} \text{ versus } 2.8 ± 1.48 \times 10^9/\text{ml}, \text{ respectively; } p = 0.002 \); \([51\text{-}100 \text{ nm}]: 3.71 ± 1.08 \times 10^9/\text{ml} \text{ versus } 2.52 ± 1.07 \times 10^9/\text{ml}, \text{ respectively; } p = 0.002 \); \([101\text{-}150 \text{ nm}]: 4.71 ± 1.92 \times 10^9/\text{ml} \text{ versus } 3.38 ± 0.9 \times 10^9/\text{ml}, \text{ respectively; } p = 0.001 \)).

Cellular origin of circulating MPs

MP cellular origin was determined by flow cytometry using monoclonal antibodies specific for the lineage markers CD41 (platelet), CD144 (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 to 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 ± 0.9 % 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 infrequent (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 (25 – 75 % interquartile range); 7 (5 – 10) pg /10^6 MPs versus 8 (4 – 14) pg/10^6 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, Table S1). 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) but individually, C18: 2n6 (linoleic acid) was elevated in PCOS subjects compared to healthy controls <0.01.

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 1,600 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 2). qPCR was used to validate the differentially expressed miR 4700-5p. Women with PCOS displayed a threefold-elevated expression of miR 4700-5p compared to 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 characterise 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 to controls (Koiou et al., 2011 and Koiou et al., 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 (<150nm), exosomal range. This may suggest selective stimulation of the intracellular classical exosomal pathway compared to 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 (Feng et al., 2010, Koga et al., 2005, Tramontano et al., 2010) including those with end-organ damage (Omoto et al., 1999). Metabolic syndrome, a disorder underpinned by insulin insensitivity, is also characterised by an increased circulating MP concentration compared to healthy controls (Arteaga et al., 2006, Agouni et al., 2008, Agouni et al., 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 (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 (Sinauridze 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 sub-population 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 recognised as a cause of hyperandrogenic PCOS (Martínez-García, 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 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


Funding

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

Conflict of Interest

The authors have no conflicts of interest to declare.
A. Phenotypic Variation in


Feng El Ehrmann D, Sturis J, Byrne M, T K, Rosenfield R and Polonsk

Connolly KD, Willis GR, Datta DBN, Ellins EA, Ladell K, Pr

Carmina E, Longo MCCRA, Rini GB and Lobo R

Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A and Boulanger CM. Circulating Endothelial Microparticles Are Associated with


Arteaga RB, Chirinos JA, Soriano AO, Wenche J, Horstman L, Jimenez JJ, Mendez A, Ferreira A, de Marchena E and Ahn YS. Endothelial Microparticles and Platelet and

Leukocyte Activation in Patients With the Metabolic Syndrome. *American Journal of Cardiology* 2006; 98(1): 70–74


*Circulation Research* 2014;114(1):109-13

Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A and Mallat Z.


Carmina E, Longo MCCRA, Rini GB and Lobo RA. Phenotypic Variation in


El-Kannishy G, Kamal S, Mousa A, Saleh O, Badrawy AE, Farahaty RE and Shokeir T. Endothelial function in young women with polycystic ovary syndrome (PCOS): Implications of body mass index (BMI) and insulin resistance. *Obesity Research & Clinical Practice* 2010; 4:e49-e56.


Garbus J, De Luca HF, Loomans ME and Strong FM. The rapid incorporation of phosphate into mitochondrial lipids. *Journal of Biological chemistry* 1963; 238:59-63

19

Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in
patients with gastric cancer: possible role of a metastasis predictor. European Journal of

Koga H, Sugiyama S, Kugiyama K, Watanabe K, Fukushima H, Tanaka T, Sakamoto T,
Yoshimura M, Jinnouchi H and Ogawa H. Elevated levels of VE-cadherin-positive
endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery

Koiou E, Tziomalos K, Katsikis I, Kalaitzakis E, Kandaraki EA, Tsourdi EA, Delkos D,
Papadakis E and Panidis D. Elevated levels of VE-cadherin-positive
endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery

Koiou E, Tziomalos K, Katsikis I, Papadakis E, Kandaraki EA and Panidis D. Platelet-
derived microparticles in overweight/obese women with polycystic ovary syndrome.

Elevated Levels of Shed Membrane Microparticles With Procoagulant Potential in the
Peripheral Circulating Blood of Patients With Acute Coronary Syndromes. Circulation
2000; 101:841-843.

Martínez-García MA, San-Millán JL and Escobar-Morreale HF. The R453Q and D151A
polymorphisms of Hexose-6-Phosphate Dehydrogenase Gene (H6PD) influence the
polycystic ovary syndrome (PCOS) and obesity. Gene 2012; 497:38-44.

Morgan CL, Jenkins-Jones S, Currie CJ and Rees DA. Evaluation of Adverse Outcome in
Young Women with Polycystic Ovary Syndrome Versus Matched, Reference Controls: A
Retrospective, Observational Study. Journal of Clinical Endocrinology & Metabolism
2012; 97:3251-3260.

Nieuwland R, Berckmans RJ, McGregor S, Böing AN, Th. M. Romijn FPH, Westendorp
RGI, Hack CE and Sturk A. Cellular origin and procoagulant properties of

Effects of Severe Hypertension on Endothelial and Platelet Microparticles. Hypertension
2003; 41:211-217.

Lombardi G and Colao A. Early Impairment of Endothelial Structure and Function in
Young Normal-Weight Women with Polycystic Ovary Syndrome. Journal of Clinical
Endocrinology & Metabolism 2004; 89:4588-4593.

Identification of microRNAs in human follicular fluid: characterization of microRNAs
that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in
vivo. Journal of Clinical Endocrinology & Metabolism 2013; 93(7); 3068-3079

Shah MD, Bergeron AL, Dong JF and López JA. Flow cytometric measurement of

Sinairidze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV and
Ataullakhanov FI. Platelet microparticle membranes have 50- to 100-fold higher specific
procoagulant activity than activated platelets. Journal of Thrombosis and Haemostasis


<table>
<thead>
<tr>
<th></th>
<th>Polycystic ovary syndrome (17)</th>
<th>Healthy Controls (18)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31 ± 6</td>
<td>31 ± 7</td>
<td>0.9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78 ± 21</td>
<td>76 ± 15</td>
<td>0.68</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30 ± 6</td>
<td>29 ± 6</td>
<td>0.61</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>91 ± 15</td>
<td>86 ± 13</td>
<td>0.31</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>111 ± 16</td>
<td>106 ± 12</td>
<td>0.24</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>1.4 ± 0.6</td>
<td>0.9 ± 0.6</td>
<td>0.02</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>1.25 (0.24 - 21.8)</td>
<td>0.9 (0.17 - 16.73)</td>
<td>0.73</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.6 ± 1.3</td>
<td>4.8 ± 1.1</td>
<td>0.67</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.2 ± 1.4</td>
<td>1.0 ± 0.5</td>
<td>0.52</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>2.4 ± 1.4</td>
<td>2.5 ± 1.3</td>
<td>0.79</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 ± 0.5</td>
<td>1.3 ± 0.6</td>
<td>0.65</td>
</tr>
<tr>
<td>Insulin AUC (nmol min/l)</td>
<td>81 ± 46.7</td>
<td>53 ± 29.6</td>
<td>0.04</td>
</tr>
<tr>
<td>Glucose AUC (mmol min/l)</td>
<td>764 ± 217</td>
<td>692 ± 133</td>
<td>0.24</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2 ± 0.9</td>
<td>2.5 ± 2.44</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Table 1. Demographic, anthropometric and metabolic characteristics of the study population. 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.
<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, hsa-miR-3689c</td>
<td>1.11</td>
<td>0.009</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>
</table>

Table 2. Differentially expressed microRNAs (miRs) in circulating microparticles. Fold change was calculated as average polycystic ovary syndrome miR expression / average healthy control miR expression. All samples tested had total miR counts >500.
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Microparticle (MP)</th>
<th>Plasma</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>C14 :0*</td>
<td>0.93%</td>
<td>0.60%</td>
<td>0.67%</td>
</tr>
<tr>
<td>C14: 1*</td>
<td>0.20%</td>
<td>0.21%</td>
<td>0.09%</td>
</tr>
<tr>
<td>C16: 0*</td>
<td>27.32%</td>
<td>5.82%</td>
<td>20.80%</td>
</tr>
<tr>
<td>C16: 1</td>
<td>0.56%</td>
<td>0.64%</td>
<td>0.78%</td>
</tr>
<tr>
<td>C16: 1n7</td>
<td>1.59%</td>
<td>2.15%</td>
<td>1.82%</td>
</tr>
<tr>
<td>C18: 0*</td>
<td>12.95%</td>
<td>4.07%</td>
<td>7.59%</td>
</tr>
<tr>
<td>C18: 1n9*</td>
<td>30.69%</td>
<td>5.58%</td>
<td>22.07%</td>
</tr>
<tr>
<td>C18 1n7*</td>
<td>0.36%</td>
<td>1.73%</td>
<td>1.67%</td>
</tr>
<tr>
<td>C18 2n6*</td>
<td>12.72%</td>
<td>6.04%</td>
<td>28.20%</td>
</tr>
<tr>
<td>C18 3n6*</td>
<td>0.20%</td>
<td>0.20%</td>
<td>0.42%</td>
</tr>
<tr>
<td>C18: 3n3*</td>
<td>0.51%</td>
<td>0.43%</td>
<td>0.74%</td>
</tr>
<tr>
<td>C20: 0</td>
<td>0.56%</td>
<td>0.53%</td>
<td>0.93%</td>
</tr>
<tr>
<td>C20: 1</td>
<td>0.06%</td>
<td>0.16%</td>
<td>0.00%</td>
</tr>
<tr>
<td>C20: 2n6*</td>
<td>0.95%</td>
<td>0.61%</td>
<td>1.67%</td>
</tr>
<tr>
<td>C20: 4n6</td>
<td>5.74%</td>
<td>3.85%</td>
<td>6.83%</td>
</tr>
<tr>
<td>C20: 5n3*</td>
<td>0.31%</td>
<td>0.25%</td>
<td>0.77%</td>
</tr>
<tr>
<td>C22: 0*</td>
<td>1.40%</td>
<td>0.95%</td>
<td>0.48%</td>
</tr>
<tr>
<td>C22: 3n3</td>
<td>0.39%</td>
<td>0.55%</td>
<td>0.23%</td>
</tr>
<tr>
<td>C22: 3n6</td>
<td>0.23%</td>
<td>0.25%</td>
<td>0.25%</td>
</tr>
<tr>
<td>C22: 5n3</td>
<td>0.34%</td>
<td>0.32%</td>
<td>0.44%</td>
</tr>
<tr>
<td>C22: 6n3*</td>
<td>0.86%</td>
<td>0.68%</td>
<td>1.83%</td>
</tr>
<tr>
<td>C24: 0</td>
<td>0.51%</td>
<td>1.06%</td>
<td>0.33%</td>
</tr>
<tr>
<td>C24: 1n9*</td>
<td>0.33%</td>
<td>0.27%</td>
<td>0.70%</td>
</tr>
</tbody>
</table>

Table S1. Comparison between microparticle (MP) and plasma fatty acid composition (% of total peak area). Mean and standard deviation (SD) values represent data from across both subject groups. * denotes significance.
Legends for figures

Fig 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.

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