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The effects of empagliflozin vs metformin on endothelial microparticles in overweight/obese women with polycystic ovary syndrome

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Abstract

Context: Endothelial microparticles (EMPs) are novel, surrogate biomarkers of endothelial function and have been shown to be elevated in women with polycystic ovary syndrome (PCOS). It remains poorly understood how pharmacological options for managing PCOS affect EMP levels.

Objective: To characterise and compare the effects of empagliflozin vs metformin on the circulating levels of EMPs in overweight/obese women with PCOS.

Methods: This was a randomised, comparative, 12-week single-centre trial conducted at the Academic Diabetes, Endocrinology and Metabolism Research Centre, Hull, UK. This analysis includes data from 39 overweight/obese women with PCOS who completed the study and were randomised to empagliflozin (15 mg/day) (n = 19) or metformin (1500 mg/day) (n = 20). Blood samples were collected at baseline and 12 weeks after treatment and analysed for specific surface proteins (ICAM-1, VCAM-1, PECAM-1, E-selectin and endoglin) expressed by circulating EMPs using flow cytometry.

Results: In the empagliflozin group, ICAM-1 (P = 0.006), E-selectin (P = 0.016) and VCAM-1 (P = 0.001) EMPs increased significantly following 12 weeks of treatment, but no changes were seen in PECAM-1 (P = 0.93) or endoglin (P = 0.13) EMPs. In the metformin group, VCAM-1 EMPs (P < 0.001) increased significantly after 12 weeks of treatment, whereas all other EMPs remained unchanged. When data were expressed as percentage change from baseline in each group, no significant differences were seen between groups for any biomarker (P-values from 0.22 to 0.80).

Conclusions: Short-term administration of empagliflozin and metformin in overweight/obese women with PCOS appear to increase EMPs expressed by endothelial cells during their activation.

Key Words

- endothelial microparticles
- polycystic ovary syndrome
- SGLT2 inhibitors
- empagliflozin
- metformin

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Introduction

Polycystic ovary syndrome (PCOS) is a common endocrinopathy, affecting up to one in five women of reproductive age (1). In addition to its cardinal features of hyperandrogenism, menstrual irregularities and polycystic ovaries, PCOS is associated with obesity, insulin resistance, dyslipidaemia and chronic systemic inflammation, all of which put women with PCOS at risk of cardiovascular (CV) disease (1, 2). Indeed, there is evidence that women with PCOS are at risk of developing subclinical atherosclerosis even at a young age (3, 4). Endothelial dysfunction is an early indicator of the atherosclerotic process, and several studies have demonstrated abnormal endothelial function assessed by flow-mediated dilation and peripheral arterial tonometry (Endo-PAT) in PCOS (5, 6, 7). Treatments that enhance endothelial function can potentially delay the progression of atherosclerosis and, ultimately, lessen the risk of future CV events (8).

Endothelial microparticles (EMPs) are extracellular vesicles formed by membrane blebbing of activated or apoptotic endothelial cells and packaging of proteins, some of which are established markers of endothelial injury such as PECAM-1, intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, endoglin and vascular endothelial growth factor (VEGF) (9, 10, 11, 12, 13). Techniques to measure EMPs rely on the preparation of platelet-free plasma and subsequent identification of cell-surface proteins (e.g. PECAM, ICAM-1 and E-selectin) (13). In addition to their microparticle-bound form, these cell-surface proteins can also be measured in their soluble from in serum samples (13). EMPs synthesis is stimulated by inflammatory cytokines, reactive oxygen species (ROS), lipopolysaccharides, thrombin and low shear stress. In turn, by facilitating the interactions of endothelial cells with immune cells, EMPs are involved in endothelial cell modifications, inflammation, coagulation and angiogenesis (9, 10, 11, 12, 13). Elevated EMP levels have been associated with cardiovascular and autoimmune diseases, cancer, endocrine and metabolic disorders and, therefore, they are often used as surrogate markers of endothelial dysfunction in these conditions (14). Compared to controls, women with PCOS have increased levels of circulating MPs deriving from endothelial cells, platelets and leukocytes (15, 16, 17, 18). Lifestyle interventions (e.g. exercise and dietary energy restriction) may be promising in reducing inflammatory markers including EMPs assessed in platelet-free plasma or their cell-surface proteins measured in serum (19, 20). Few available studies on the effects of pharmacological agents for PCOS management on the EMP levels have yielded mixed results. For example, hormonal contraceptives, which are often prescribed to manage hyperandrogenism and menstrual disturbances in this population, have been shown to increase serum ICAM-1 (21). Metformin, which may exert beneficial effects on insulin resistance and hyperinsulinaemia in PCOS (22), has been shown to reduce ICAM-1 (23), VCAM-1 (18), E-selectin (23) and tissue factor levels in serum samples (24). Similarly, our recent study showed that treatment with exenatide resulted in reductions in serum ICAM-1, p-selectin and e-selectin, although no pronounced changes were seen in endothelial function (25).

Empagliflozin is a sodium-glucose cotransporter 2 (SGLT-2) inhibitor used in the treatment of type 2 diabetes. SGLT-2 inhibitors act by inhibiting glucose reabsorption by the kidney and, therefore, they result in mild glycosuria and net caloric loss (26). SGLT-2 inhibitors have been demonstrated to reduce weight, improve arterial stiffness and vascular resistance and decrease the relative risk for cardiovascular and all-cause mortality in patients with type 2 diabetes (27, 28). In the first study comparing the effects of empagliflozin and metformin in overweight/obese women with PCOS, we demonstrated that empagliflozin treatment over 12 weeks resulted in significant improvements in anthropometric parameters and body composition, without overt changes in hormonal or metabolic parameters (29).

The aim of this analysis was to explore and compare the effects of empagliflozin and metformin on a panel of EMPs bearing proteins with established roles in endothelial injury (13) in overweight/obese women with PCOS. Given that empagliflozin and metformin have been shown to exert positive effects on cardiovascular risk factors (27, 28, 30, 31, 32), with these effects potentially mediated by amelioration in inflammation and endothelial injury (30, 31, 32, 33, 3, 35, 36), we hypothesised that both treatments would result in reductions in EMP levels

Materials and methods

This is a secondary analysis of an open-label, randomised study which compared the effects of empagliflozin and metformin in overweight/obese women with PCOS. This study was approved by the Medicines and Healthcare Products Regulatory Authority (MHRA) (Ref: 21411/0254/001-0001), the Yorkshire & Humber Health Research Authority and Leeds East Research Ethics

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Committee (REC reference: 17/YH/0118), registered at www.clinicaltrials.gov (NCT03008551) and conducted in the Academic Diabetes, Endocrinology and Metabolism Research Centre at Hull Royal Infirmary. All participants gave their informed consent in writing.

Inclusion/exclusion criteria and study procedures have been explained in detail (29). In brief, all women aged between 18 and 45 years, had a BMI ≥25 kg/m^2 and were diagnosed with PCOS according to the Rotterdam criteria (37). Exclusion criteria were differential diagnoses of non-classical 21-hydroxylase deficiency, hyperprolactinaemia, Cushing's disease and androgen-secreting tumours, pregnancy or intention to become pregnant, breastfeeding, documented use of oral hormonal contraceptives and hormone-releasing implants, metformin or other insulin-sensitizing medications, clomiphene citrate or oestrogen modulators, gonadotropin-releasing hormone modulators and Minoxidil, diagnosis of diabetes, history or presence of malignant neoplasms within the last 5 years, pancreatitis (acute or chronic), recurrent urinary tract infections or gastrointestinal tract surgery, ongoing, inadequately controlled thyroid disorder and known hypersensitivity to the investigational medicinal products or any of their excipients.

PCOS patients received either empagliflozin 25 mg (Jardiance) or metformin SR 1500 mg (Bolamyn) per day over a 12-week period. Evaluations were performed at baseline and after 12 weeks of treatment. Examination included anthropometric (weight, BMI, waist circumference (WC) and hip circumference (HC)) and body composition assessments and an endothelial function measurement – detailed description of these measurements is provided in 29. Blood samples were collected at these time points and analysed for EMPs. Additional biochemical analyses included measurement of reproductive hormones and cardio-metabolic parameters (fasting glucose, fasting insulin, HOMA-IR, total cholesterol, LDL-C, HDL-C, triglycerides (TG) and hs-CRP): these results have been presented in our previous work (29).

Endothelial function was assessed using Endo-PAT 2000 (Itamar Medical Ltd, Caesarea, Israel). Participants relaxed for at least 15 min in a quiet, controlled temperature (22–24°C) room. Endo-PAT biosensors were positioned on the index fingers of both hands. For this assessment, subjects were asked to avoid talking or moving. The probes were inflated, and the signals were recorded on the computer according to manufacturer's instructions. The assessment comprised of: (1) baseline recording (0–5 min), (2) blood pressure cuff inflation to a supra-systolic level (at least 60 mmHg above systolic pressure and not ≥200 mmHg) (5–10 min), and (3) blood pressure cuff deflation and recording of Endo-PAT readings (10–15 min). Output variables included the reactive hyperaemia index (RHI), a measure for endothelial function and the augmentation index (AI), a measure for arterial stiffness.

EMP assessment and characterisation

To prepare platelet-free plasma (within 2 h of blood drawing), blood samples were centrifuged at 1000 ***g*** for 10 min and the supernatant was further centrifuged at 12,000 ***g*** for 10 min. All assays were performed on a BD Accuri™ C6 Plus flow cytometer (BD Biosciences). The platelet-free plasma samples (25 µL) together with 5 µL of fluorescin isothyocynate conjugated monoclonal antibodies against cell-type specific antigens were incubated for 30 min in darkness. EMPs were identified using platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) (BD Biosciences); intercellular adhesion molecule 1 (ICAM-1 or CD54) (Bio-Rad), E-selectin (or CD62-E) (Bio-Rad); endoglin (or CD105) (BD Biosciences) and vascular cell adhesion molecule 1 (VCAM-1 or CD106) (BD Biosciences). After incubation, the samples were diluted in 300 µL of PBS that had been filtered through a sterile 0.1-µm syringe filter (Minisart™, Nottingham, UK). A total of 25 µL of counting beads with an established concentration (AccuCheck Counting Beads, Life Technologies Corporation) were added to each sample to calculate EMPs as absolute numbers per microliter.

Statistical analysis

All variables were checked for normality using the Shapiro–Wilk test and for extreme outliers (>3 times interquartile range (IQR) above the third quartile or <3 times IQR below the first quartile) graphically. Participants indicated as extreme outliers for each EMP were excluded from analysis. Within-group comparisons between baseline and 12-week follow-up were performed using a paired *t*-test or a signed-rank test for normally and non-normally distributed data. For between-group comparisons, data were expressed as percentage change from baseline and analysed using an independent *t*-test or Mann–Whitney *U*-test for normally and non-normally distributed data. Correlations between changes in EMPs and Endo-PAT measures were examined using Spearman's correlations. Values are presented as median and interquartile range. Two-tailed analyses were performed using IBM-SPSS version 24.0 with statistical significance set at $P \leq 0.05$.

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Results

The baseline anthropometric characteristics, hormonal and metabolic profile of the participants in the empagliflozin ($n=19$, age: 26.0 (8.0) years, BMI: 37.1±6.2 kg/m^2) and the metformin ($n=20$, age: 31.5 (9.0) years, BMI: 38.7±7.8 kg/m^2) groups have been presented previously (29).

In the empagliflozin group, ICAM-1 ($P=0.006$), E-selectin ($P=0.016$) and VCAM-1 ($P=0.001$) EMPs increased significantly following 12 weeks of treatment, but no changes were seen in PECAM-1 ($P=0.93$) or endoglin ($P=0.13$) EMPs (Table 1). In the metformin group, VCAM-1 ($P<0.001$) EMPs significantly increased after 12 weeks of treatment, whereas all other EMPs remained unchanged (Table 1).

When data were expressed as percentage change from baseline in each group, no significant differences were seen between the treatment groups for any marker (P-values from 0.22 to 0.80) (Table 1.).

As we reported in 29, endothelial function determined by Endo-PAT did not change following treatment with empagliflozin (RHI; baseline: 1.6 (0.5), 12 weeks: 1.5 (0.7), AI; baseline: −3.0 (17.0), 12 weeks: −4.0 (20.0)) or metformin (RHI; baseline: 1.7 (0.6), 12 weeks: 1.6 (0.5), AI; baseline: 0.5 (14.0), 12 weeks: 0.5 (19.5)). There were no significant correlations between EMP changes and changes in Endo-PAT measures in the empagliflozin or metformin group (all P values >0.05).

Discussion

This study characterised and compared the effects of empagliflozin vs metformin on EMPs in overweight/obese women with PCOS. Contrary to our hypothesis, within-group comparisons revealed increases in ICAM-1, E-selectin and VCAM-1 EMPs following a 12-week treatment period with empagliflozin, whereas treatment with metformin resulted in increases in VCAM-1 EMPs only. Between groups comparisons did not show any differences in any of these markers, suggesting a similar pattern of changes in both treatment arms. These results consistently indicate activation of endothelial cells with empagliflozin and metformin.

Few studies on the impact of pharmacological management options for PCOS on EMP levels have yielded mixed results. Diamanti-Kandarakis *et al.* showed a reduction in soluble VCAM-1 levels independent of BMI changes after 6 months of metformin administration (1700 mg/day) (18). In the same study, metformin did not result in changes in soluble ICAM-1 and E-selectin (18). In contrast, reduction in serum ICAM-1 and E-selectin were reported in a 12-week intervention with metformin (increasing daily dose from 500 to 1500 mg) (23). A cross-sectional study demonstrated lower total MPs and tissue factor in women with PCOS using metformin (2×850 mg/day) for at least 6 months (24). These discrepant results may be largely due to differences in metformin treatments (i.e. duration) and participants' characteristics (age, BMI, insulin resistance or other metabolic conditions). Importantly, the results from these previous investigations (18, 23, 24) are not directly comparable to our findings. This is because we assessed changes in EMPs bearing PECAM-1, ICAM-1, E-selectin, endoglin and VCAM-1, rather than changes in the serum concentrations of these surface proteins. The increase in VCAM-1 EMPs following 12 weeks of treatment with metformin in our study suggests that VCAM-1 may be selectively packaged into EMPs at the cost of its soluble release or that the VCAM-1 expression on the endothelial cells is increased, thus increasing the probability of VCAM-1 becoming incorporated into EMPs. If soluble VCAM-1 is decreased (18), this may be due to preferential VCAM-1 packaging into EMPs. We have previously shown that the Endoglin:VCAM-1 EMP ratio was shifted to a more VCAM-1 dominant profile in women with PCOS (38).

Table 1 Changes in circulating EMPs following 12 weeks of treatment with empagliflozin and metformin.

	Empagliflozin (n = 19)			**Metformin** (n = 20)		
	Baseline	12 weeks	%baseline change	Baseline	12 weeks	%baseline change
PECAM-1	536 (274)[c]	581 (242)	6.9 (72.3)[c]	529 (171)[b]	426 (336)	−13.7 (69.3)
ICAM-1	999 (717)[c]	1597 (931)[a]	52.3 (106.1)[c]	785 (872)[b]	1351 (787)	54.9 (150.5)
E-selectin	681 (460)[c]	1055 (520)[a]	52.2 (103.8)[c]	677 (502)	935 (511)	32.2 (114.5)
Endoglin	153 (91)[c]	120 (63)	−28.5 (61.2)[c]	132 (39)[b]	106 (87)	−19.0 (60.6)
VCAM-1	199 (179)	426 (143)[a]	62.6 (102.2)	229 (98)	451 (267)[a]	82.2 (99.0)

Data are presented as median (interquartile range).
[a]$P<0.05$, significant difference from baseline within treatment group; [b]Analysis performed in 19 participants; [c]Analysis performed in 18 participants.
ICAM-1, intercellular adhesion molecule 1; PECAM-1, endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule 1.

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There are no comparative studies on the effects of empagliflozin on EMP levels in PCOS. A study using another SGLT-2 inhibitor, canagliflozin, did not result in alterations in serum VCAM-1 levels in patients with type 2 diabetes (39). In the present analysis, the simultaneous increases in ICAM-1, E-selectin and VCAM-1 EMPs indicate endothelial cell activation and enhanced interactions between endothelial and other immune cells. Specifically, ICAM-1 is an adhesion molecule, which regulates vascular permeability by facilitating leukocytes rolling within vasculature and by promoting leukocytes-endothelium interrelationships (40). E-selectin allows the binding of neutrophils, monocytes and T-cell subpopulations to sites of vascular injury and promotes angiogenesis. VCAM-1 is another adhesion molecule, which mediates leukocytes rolling and adhesion to the endothelium, regulates leukocytes transendothelial migration and modulates endothelial signalling through the activation of NADPH oxidase and formation of ROS (41, 42).

Increasing evidence suggests that the role of EMPs is more complex than initially perceived and that it is unclear whether EMPs-mediated alterations are part of physiological vascular homeostasis or if they contribute to pathological conditions (10, 11, 12). In our study, there was no evidence of endothelial impairment (Endo-PAT) in response to treatment with empagliflozin or metformin. As such, it is uncertain whether the increases in endothelial markers represent a transitory, adaptive response to regenerate the endothelium, limit vascular damage and restore homeostasis or if they contribute to endothelial dysfunction and increased CVD risk in the longer-term (11). The discordant results of the adhesion molecules assessed in serum and the direct measures of endothelial measures have been reported previously (25). Our findings indicate that biochemical markers of endothelial function change early during an intervention, whereas functional measures may need more time to be altered. Longer-time studies with concurrent measurements of endothelial markers and direct measures of endothelial function are required to provide further insights into the relationship of these assessments.

Although the long-term CVD risk following empagliflozin and metformin treatment remains to be determined in PCOS, studies using these drugs support cardioprotective effects in patients without PCOS. The EMPA-REG OUTCOME trial revealed that empagliflozin given in addition to standard care reduced the risk of CV death by 38% and heart-failure hospitalisation by 35% in patients with type 2 diabetes (27). Among other mechanisms, the authors mentioned that improvements in arterial stiffness may contribute to this CVD risk reduction (27). Similar cardio- and reno-protective effects relative to placebo were demonstrated after treatment with canagliflozin in patients with type 2 diabetes and a history of CVD or >2 CVD factors (43). In agreement with human studies, preclinical studies in diabetic or prediabetic animals have demonstrated reductions in oxidative stress, inflammatory cytokines and vascular dysfunction following treatment with SGLT-2 inhibitors (33, 34, 35, 36). Similarly, an increasing number of studies support that metformin exerts cardioprotective effects in patients with and without diabetes (30). Mechanistic studies have demonstrated that the cardioprotective effects of metformin may be explained by its reducing endoplasmic reticulum (ER) and oxidative stress effects, anti-atherogenic, anti-inflammatory action, protection from endothelial injury and favourable effects on blood lipids (30, 31, 32).

The current analysis is strengthened by its design and the assessment of a panel of EMPs indicative of endothelial cell activation and apoptosis. This study was powered to detect difference in *hs*-CRP (29); therefore, a limitation of the present analysis is the lack of pre-specified statistical testing due to its post-hoc nature. Data from a control group would be informative on whether EMP levels change due to PCOS *per se*; however, such data are unavailable in this analysis. Given the high prevalence of menstrual-cycle disorders in women with PCOS, including amenorrhea and oligomenorrhea, we were unable to adjust for this parameter in the current intervention. Although there is the suggestion that EMP may be affected by the menstrual cycle (44), this requires clarification and confirmation. The assessment of additional markers indicative of endothelial function and systemic inflammation (endothelin-1, angiotensin II, ROS, TNF (TNF-a) or interleukin (IL)-1 or 6) were not performed as part of this study, but their future evaluation could provide insights into the mechanism actions of metformin and empagliflozin in this population. Finally, the duration of the present study is short (12 weeks) and it is possible that treatments over longer periods are required to have differential effects on EMP levels or direct measures of endothelial function.

Conclusions

In summary, treatment with empagliflozin and metformin for over 12 weeks in obese women with PCOS increased levels of EMPs, which are likely to be expressed by

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endothelial cells during their activation. We did not show alterations in direct measures of endothelial function (Endo-PAT) with either treatment, suggesting that surrogate markers precede or do not reflect endothelial function changes in this population. Further longer-term, placebo-controlled and comparative-treatment pharmacological trials are required to confirm these findings and elucidate the mechanisms that contribute to the observed EMP changes and their relationship with clinical outcomes in diverse PCOS populations.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Z J, E S K, S L A and T S participated in study conception and design. Z J performed the acquisition of data. Z J, M P, A S R, E S K, S L A and T S participated in analysis and/or interpretation of data. M P drafted the paper. All authors reviewed and approved the final manuscript. T S is the guarantor of the study.

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