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Androgens drive microvascular endothelial dysfunction in women with polycystic ovary syndrome: role of the endothelin B receptor

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Key points

- Polycystic ovary syndrome (PCOS) is a complex syndrome with cardiovascular risk factors, including obesity and insulin resistance.
- PCOS is also associated with high androgens, increases the risk of cardiovascular dysfunction in women. Due to the complexity of PCOS, had it has been challenging to isolate specific causes of the cardiovascular dysfunction.
- Our measure of cardiovascular dysfunction (endothelial dysfunction) was most profound in lean women with PCOS.
- The endothelin-1-induced vasodilation in these PCOS subject, was dependent on the ET_BR but was not NO-dependent.
- We also demonstrated oestrogen administration improved endothelial function in lean and obese women with PCOS likely because oestrogen increased NO availability.
- Our studies indicate a primary role for androgens in cardiovascular dysfunction in PCOS.

Abstract Endothelin-1 (ET-1) is an indicator of endothelial injury and dysfunction and is elevated in women with androgen excess polycystic ovary syndrome (AE-PCOS). The endothelin B receptor (ET_BR) subtype mediates vasodilatation, but is blunted in women with PCOS. We hypothesized that androgen drives endothelial dysfunction in AE-PCOS women and oestradiol (EE) administration reverses these effects. We assessed microvascular endothelial function in women with (7 lean and 7 obese) and without AE-PCOS (controls, 6 lean, 7 obese). Only obese AE-PCOS women were insulin resistant (IR). We evaluated cutaneous vascular conductance (%CVC_{max})

Charlotte Usselman's research focuses on the impact of sex and sex hormones on cardiovascular function and health in humans. Following a two-year American Heart Association-funded postdoctoral Fellowship under the mentorship of Dr Nina Stachenfeld at the John B. Pierce Laboratory and the Yale School of Medicine, Dr Usselman accepted an Assistant Professor position at McGill University in the Department of Kinesiology and Physical Education. She continues her research into cardiovascular co-morbidities of PCOS in her laboratory at McGill, focusing on the neurovascular sequelae of the disorder. She also studies neurovascular regulation in young, postmenopausal, and previously preeclamptic women.

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with laser Doppler flowmetry during low dose intradermal microdialysis ET-1 perfusions (1, 3, 4, 5 and 7 pmol) with either lactated Ringer solution alone, or with ET_BR (BQ-788), or nitric oxide (NO) inhibition (L-NAME). Log[ET-1]–%maxCVC dose–response curves demonstrated reduced vasodilatory responses to ET-1 in lean AE-PCOS ($logED_{50}$, 0.59 ± 0.08) *versus* lean controls ($logED_{50}$, 0.49 ± 0.09, $P < 0.05$), but not compared to obese AE-PCOS ($logED_{50}$, 0.65 ± 0.09). ET_BR inhibition decreased ET-1-induced vasodilatation in AE-PCOS women ($logED_{50}$, 0.64 ± 0. 22, $P < 0.05$). This was mechanistically observed at the cellular level, with ET-1-induced, DAF-FM-measurable endothelial cell NO production, which was abrogated by dihydrotestosterone in an androgen receptor-dependent manner. EE augmented the cutaneous vasodilating response to ET-1($logED_{50}$ 0.29 ± 0.21, 0.47 ± 0.09, $P < 0.05$ for lean and obese, respectively). Androgens drive endothelial dysfunction in lean and obese AE-PCOS. We propose that the attenuated ET-1-induced vasodilatation in AE-PCOS is a consequence of androgen receptor-mediated, suppressed ET_BR-stimulated NO production, and is reversed with EE.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common reproductive endocrinopathy (Barontini *et al.* 2001), affecting ∼1 in 10 women of reproductive age (Tsilchorozidou *et al.* 2004), and is the leading cause of female infertility (Goodarzi *et al.* 2011; Jayasena & Franks, 2014). Approximately 75% of women with PCOS have the more severe reproductive and metabolic PCOS phenotype that is dominated by features of hyperandrogenism. Androgen excess (AE)-PCOS is a complex reproductive disorder also associated with insulin resistance (IR), obesity, hyperlipidaemia and cardiovascular disease (CVD), including hypertension and endothelial dysfunction (Diamanti-Kandarakis *et al.* 2001; Wenner *et al.* 2011*a*, 2013). Endothelial dysfunction is a known early marker of (and probable causative factor in) atherosclerosis and coronary artery disease (Kravariti *et al.* 2005; Vanhoutte, 2009). We have shown compromised endothelial-mediated vasodilatation in the microcirculation in young women with AE-PCOS (Wenner *et al.* 2011*a*, 2013) and others have shown endothelial dysfunction in the brachial artery using flow-mediated vasodilatation (Diamanti-Kandarakis *et al.* 2001). As such, individuals with AE-PCOS are predisposed to early onset CVD. Although young women with AE-PCOS commonly manifest a spectrum of CVD risk markers, including obesity, IR and hypertension, androgen excess itself is a primary driver of endothelial dysfunction (Wenner *et al.* 2013; Hurliman *et al.* 2015).

Plasma endothelin-1 ($[ET\text{-}1]_P$), an important indicator of endothelial injury, is increased in women with AE-PCOS (Yanagisawa *et al.* 1988; Inoue *et al.* 1989). Secreted through the basolateral compartment of endothelial cells, ET-1 has vasoconstrictor actions on the vascular smooth muscle in the peripheral circulation via two primary receptor subtypes, ET_AR and ET_BR (Ariai *et al.* 1990; Lin *et al.* 1991). The ET_BR is also expressed in the endothelium (Ishikawa *et al.* 1994) where it mediates vasodilatation (Verhaar *et al.* 1998; Kellogg *et al.* 2001) through nitric oxide (NO) and prostacyclin production (de Nucci *et al.* 1988; Tsukahara *et al.* 1994; Verhaar *et al.* 1998). Androgen suppression improves endothelium-mediated microvascular responsiveness and increases vasodilator tone in women with AE-PCOS, with increased circulating ET-1 mediated through the ET_BR (Wenner *et al.* 2013). When expressed on normally functional endothelial cells, ET-1 stimulates NO production when engaging ET_BRs (Ishikawa *et al.* 1994; Zhou *et al.* 2004; Bourque *et al.* 2011). Further, sex hormones influence the function of ET-1, ET_AR and ET_BR in the peripheral circulation (Wenner *et al.* 2011*a*, 2013).

A primary feature of normally functioning endothelium is the production of NO in response to a diverse array of agonists. In AE-PCOS the androgen receptor (AR) may result in impaired, agonist-triggered endothelial NO release and/or NO responsiveness. We believe this to be a key causative link between AE-PCOS, endothelial dysfunction and ultimately cardiovascular disease. However, the mechanism of AR-mediated endothelial dysfunction is not defined, nor is the array of agonist-triggered, eNOS-activating receptors negatively influenced by androgens. We therefore directly assessed androgen receptor engagement-mediated effects on ET-1-induced NO production in an isolated endothelial cell (EC) culture model.

In order to understand the effects of androgens on the cardiovascular system in AE-PCOS, it is necessary to isolate androgen exposure from the other CVD risk factors in women with AE-PCOS. Therefore, to examine the relative impact of hyperandrogenism, IR and obesity on endothelial function in AE-PCOS, we recruited women

Table 1. Subject characteristics

	Lean		Obese	
	Control	AE-PCOS	Control	AE-PCOS
Age (years)	26 ± 7	27 ± 5	25 ± 4	30 ± 4
Weight (km)	61 6 ± 9.0	62.0 ± 3.7	97.1 ± 17.8†	102.2 ± 15†
Height (cm)	168.4 ± 12.2	163.9 ± 6.5	159.8 ± 2.4	161.7 ± 2.7
BMI (kg m^{-2})	21.7 ± 1.7	23.1 ± 1.6	38.1 ± 7.4†	39.1 ± 5.8†
Glucose (AUC)	19577 ± 2231	19672 ± 2110	21075 ± 49075	22209 ± 3528
Insulin (AUC)	9536 ± 4064	8059 ± 3304	12103 ± 3097	17030 ± 9329†
MAP (mmHg)	89 ± 11	88 ± 6	89 ± 9	95 ± 6
SBP (mmHg)	116 ± 14	113 ± 9	118 ± 8	124 ± 4
DBP (mmHg)	77 ± 10	78 ± 7	76 ± 10	81 ± 8

†Significantly different ($P < 0.05$) from lean PCOS or control values. Data are presented as means ± SD.

from four groups: lean and obese women with AE-PCOS, and lean and obese women without AE-PCOS. We tested the hypothesis that the androgen dominant hormonal milieu causes endothelial dysfunction independently of IR or obesity in women with AE-PCOS by reducing ET_BR function. We further hypothesized that oestradiol would reverse the androgen-driven ET-1 effects and improve endothelial function in women with AE-PCOS via improved endothelial cell and ET_BR function.

Methods

Ethical approval

All subjects gave written informed consent to participate in the study, which conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database. The study was approved by the Human Investigation Committee of Yale School of Medicine (HIC Protocol no. 1401013220).

Subjects

We recruited 14 women with AE-PCOS ($n = 14$, 7 lean and 7 obese) and 13 women without AE-PCOS (Controls, $n = 13$, 6 lean, 7 obese). All women were non-smoking and indicated excellent health other than AE-PCOS when interviewed about their medical history. Women without AE-PCOS reported regular menstrual cycles (26—32 days) with no gynaecological abnormalities.

Following enrollment in the study, all women underwent transvaginal ultrasound with an experienced obstetrician/gynaecologist to either confirm the diagnosis of PCOS or exclude PCOS and polycystic appearing ovaries in the control women. These ultrasound assessments were conducted as close to the physiological test visits as possible (i.e. within a month), although due to scheduling constraints this was not possible in all subjects (average length of time between ultrasound and physiological testing: 1.4 ± 1.7 months; range: 0–6 months). Potential controls were also excluded if they had any of the symptoms or signs of AE-PCOS (see below). Subjects with AE-PCOS were admitted into the study based on the presence of hyperandrogenism in addition to one out of two cardinal characteristics of PCOS based on the Rotterdam Criteria (Trivax & Azziz, 2007). To be characterized as AE-PCOS, at least one of the two following criteria were present: oligo/anovulation, defined as intermenstrual period of 3 ≥ 45 days, or a total of ≤8 menses per year; or have polycystic ovaries. Polycystic ovaries were defined by the morphological appearance of 12 small follicles in the range of 3–9 mm mean diameter in the ovary on day 3 as determined by transvaginal ultrasound. We excluded other disorders of the ovaries, adrenal and pituitary. Women were also excluded if they had a history of blood clots, hypertension, stroke, epilepsy, diabetes, or cancer. Subjects in both groups were not taking any medications. Subject characteristics are described in detail in Table 1.

Oral glucose tolerance test

To assess IR, all women underwent a 3-h oral glucose tolerance test (OGTT). Women reported to the laboratory in the morning after an overnight fast. They provided a urine sample to determine hydration status and underwent an over-the-counter pregnancy test. Women were seated in a semirecumbent position in a modified dental chair, and an IV catheter was placed in the left arm. After a 30-min resting period, a blood sample was taken for fasting measures of plasma glucose and insulin. Women then consumed a 75-mg glucose beverage (Orangedex; Custom Laboratories, Baltimore, MD, USA), and venous blood samples were drawn every 30 min to analyse plasma glucose and insulin concentrations ($[In]_p$). The area under the curve (trapezoid method) for $[In]_p$ concentration during the 180-min period was used to determine insulin resistance (Table 1) (Ciampelli *et al.* 2005). We defined IR

according to the American Diabetes Association (2014). Fasting and OGTT estimates of IR can be limited in ruling out IR in lean insulin-sensitive AE-PCOS (Tosi *et al.* 2017), so we used especially stringent standards to define this group. We defined subjects in both control and AE-PCOS groups as having IR with an area under the curve (AUC) of glucose/insulin $>$ 2.0 (Matsuda & DeFronzo, 1999; Lerchbaum *et al.* 2014; Morciano *et al.* 2014).

Human subject experimental protocol

To keep hormone exposure levels consistent, all women without AE-PCOS were tested in the first 5 days of a normal menstrual cycle. Most of the women with AE-PCOS were not cycling, so they were tested at their convenience. Three women with AE-PCOS reported regular menses ($n = 2$ lean, $n = 1$ obese), and were testsed in the first 5 days of a normal menstrual cycle. Subjects participated in two experiments on separate days, once prior to and once after taking 7 days of 30 μg day^{-1} ethinyl oestradiol (EE). Experiments were separated by at least one week.

Skin blood flow and microdialysis protocol

Skin blood flow studies were conducted in an environmental chamber ($T_a = 28$°C). Subjects ate a diet controlled for water and sodium the night before and the morning of the skin blood flow test under each experimental condition (∼13 kcal (kg·body weight)$^{-1}$. Upon arrival, we immediately assessed hydration state from urine specific gravity, which was between 1.009 and 1.020 in all subjects. Following the urine sample, the subject was weighed to the nearest 10 g on a beam balance and positioned in the semi recumbent position in a dental chair modified to support the forearm. During a resting period, the subjects were instrumented for the measurement of beat-to-beat arterial blood pressure (Pinaz method, Finometer, Finapres Medical Systems, Affligem, Belgium) and skin microdialysis (see below). After 1 h of seated rest, a blood sample was drawn to measure serum (S) concentrations of 17-β-oestradiol ($[E_2]_S$), progesterone ($[P_4]_S$), free and total testosterone ($[T_{free}]_S$, $[T_{total}]_S$) and plasma ET-1 concentration $[ET\text{-}1]_P$).

Skin blood flow. To determine microvascular endothelial function, we used laser Doppler flowmetry (Doppler Monitor, PF 5020 LDPM Unit, Perimed AB, Stockholm, Sweden), which measures red blood cell flux, an index of skin blood flow (SkBF), at three different sites on the forearm. Our Doppler probes measure both SkBF and control local skin temperature. We use laser Doppler flowmetry coupled with cutaneous microdialysis to study SkBF because this method permits the study of a local and immediate SkBF response to the drug perfusions.

Microdialysis probe placement. Under sterile conditions, three 27-gauge needles were inserted on the dorsal aspect of the forearm (intradermal). The entrance and exit sites were 2 cm apart, and the needles were 2 cm apart. Microdialysis probes were threaded through the lumen of the needle, after which the needle was removed, leaving the hollow fibre portion of the microdialysis probe in place under the skin. All three microdialysis probes were perfused with lactated Ringer solution (2 ml min^{-1}; Harvard microinfusion pump; Harvard Apparatus, Holliston, MA, USA) for 90–120 min after placement to allow for recovery from the microdialysis probe placement (Holowatz *et al.* 2008; Hodges *et al.* 2009).

Drug perfusions. Following recovery, we measured resting SkBF for 15 min at all sites. Immediately after the resting measurement, L-N^G-nitroarginine methyl ester (L-NAME) and a selective ET_BR antagonist (BQ-788; sodium *N*-{[(2R,6S)-2,6-dimethyl-1-piperidinyl]carbonyl-4-methyl-L-leucyl-*N*-[(1R)-1-carboxylatopentyl]-1-(methoxycarbonyl)-D-tryptophanamide) were perfused through probes one and two, respectively at a rate of 2 μl min^{-1} for 45 min. We used concentrations of BQ-788 previously determined in our laboratory to be effective in healthy women and in women with AE-PCOS (Wenner *et al.* 2011*a*) and concentrations of L-NAME used in the literature (Holowatz *et al.* 2005; Bruning *et al.* 2012). Following perfusion of the blocking agents, SkBF was measured again for 15 min. After perfusing the pharmacological blocking agents, increasing doses of ET-1 were continuously perfused through all three probes at a rate of 5 μl min^{-1} for 15 min at the following doses: 1, 3, 4, 5 and 7 pmol. Therefore, all microdialysis probes were progressively perfused with low doses of ET-1, with individual microdialysis probes receiving one of the two pharmacological agents, and one probe receiving ET-1 alone. At the end of the perfusions, the local heating devices (within the laser Doppler probes) were increased to 42°C to induce vasodilatation and clamped for ∼30 min and maintained until a clear plateau was reached to determine maximal SkBF. Maximal SkBF was used for subsequent analyses in order to allow for account for anatomical differences in the number of blood vessels under the different probes. Syringes for microdialysis studies were prepared the morning of the study by the Investigational Drug Service at Yale New Haven Hospital.

Blood analysis

An aliquot of blood was transferred into a tube without anticoagulant for the determination of E_2 and P_4, IN and T (free and total). Another aliquot was transferred into a tube with K^+EDTA for the determination of ET-1. Serum (S) concentrations of E_2 and P_4, T (free and total) and $[ET\text{-}1]_P$ were measured using enzyme-linked immuno-

sorbent assay (ELISA) methods. Intra- and inter-assay coefficients of variation for the low standard for $[E_2]_S$ (SEM = 180 ± 13 pg ml^{-1}) were 5.1% and 6.9% (Alpco, Salem, NH, USA), and for $[P_4]_S$ (3.5 ± 0.2 ng ml^{-1}) they were 5.1% and 8.0% (Alpco). Intra- and inter-assay coefficients of variation for the low standard for $[In]_P$ were 3.5% and 4.9% (Alpco). Intra- and inter-assay coefficients of variation for $[T_{total}]_S$ were 5.7% and 5.7% (Alpco), and $[T_{free}]_S$ were 3.9% and 4.0% (Alpco). Intra- and inter-assay coefficients of variation for the mid-range standards for [ET-1]P (17 ± 18 pg ml^{-1}) were 4.1% and 4.3% (Alpco).

In vitro NO production assay

Bovine aortic endothelial cells (BAECs, passage number 5 to 8, Lonza) were grown to confluence in DMEM (ThermoFisher) supplemented with 10% FBS (VWR) in optically clear-bottomed black gelatin-coated 96-well plates (PerkinElmer). Following two washes with PBS, the medium was replaced with Phenol Red-free DMEM (ThermoFisher) supplemented with 5% gelding horse serum (Sigma) with or without 70 nM DHT (Sigma). The final concentration of ethanol (carrier solution for DHT) was 0.002% v/v. After overnight pretreatment with DHT, cells were loaded with 5 μM DAF-FM diacetate (ThermoFisher) in the presence or absence of 1 mM L-NAME (Sigma) and stimulated with 100 nM ET-1 (Sigma) or 10 ng ml^{-1} VEGF (R&D Systems) for 15 min. DAF-FM fluorescence was measured in Synergy 2 plate reader (BioTek) using 485/25-nm bandwidth excitation filter and 528/20-bandwidth emission filter. NO-dependent DAF-FM fluorescence in the corresponding groups was calculated by subtracting the fluorescence intensity of the cells treated with L-NAME from the fluorescence intensity of the cells without L-NAME treatment and expressed as percentage of baseline fluorescence (control). All readouts were carried out in a minimum of triplicate wells for each treatment.

Data analysis

Laser Doppler flowmetry data were recorded at 1000 Hz using LabChart 8 (ADInstruments, Bella Vista, NSW, Australia). The final 2 min of SkBF and mean arterial blood pressure (MAP) at each ET-1 dose were used for analysis, and all SkBF responses were closely inspected to ensure a plateau had been reached at each level. Cutaneous vascular conductance (CVC) was calculated as mean SkBF/MAP, and expressed as a percentage of maximum (%max). Endothelin-1 doses were transformed to logarithmic concentrations, and CVC normalized so that baseline CVC = 100% (pre-ET-1 perfusion), and percentage maximal CVC (%maxCVC) at the highest ET-1 concentration = 100. We used a sigmoidal dose–response curve with variable slope, equivalent to a four-parameter logistic equation (Dodson & Rhoden, 2001), with constraints set for the bottom (zero) and top (100) parameters (Prism v 7, GraphPad Software Inc., La Jolla, CA, USA) to best fit parameters of the model (Wenner *et al.* 2011*a*,*b*). The logED50 (the dose causing 50% of the drug's maximal effects) and Hill slope (to define the sensitivity to the ET-1 stimulus) of the dose–response curves were determined by non-linear regression curve fitting of mean dose–response data fitted to the equation $Y = Y_{min} + (Y_{max} - Y_{min})/(1 + [ED_{50}/X]n)$, where Y_{min} and Y_{max} are the minimal and maximal responses, respectively, X is the ET-1 concentration, and n is the Hill slope (Prism).

Statistics

We analysed the differences in logED50 and slopes between groups and within each group across hormone conditions using Friedman's test for repeated measures comparisons (Prism). Differences in baseline characteristics between the groups were determined by independent Student's t tests and ANOVAs. Differences were considered statistically significant when $P < 0.05$. All data in tables are presented as means ± SD; data in graphs are presented as means ± SEM.

Results

General subject characteristics were similar between control and AE-PCOS groups within their respective BMI groups, but by design our obese subjects weighed more and had greater BMI than our lean subjects (Table 1). The obese AE-PCOS were IR compared to lean AE-PCOS ($P = 0.01$) based on responses to OGTT. Endothelin-1 was elevated in AE-PCOS, independently of BMI (Table 2, $P = 0.01$). Both $[T_{total}]_S$ and $[T_{free}]_S$ were greater in AE-PCOS within BMI groups compared to their respective controls (Table 2, $P = 0.02$). However, neither $[T_{free}]_S$ nor $[T_{total}]_S$ was different between lean AE-PCOS *versus* control subjects while taking EE (Table 2). None of the women experienced symptoms due to EE administration.

Skin blood flow responses

Pre-EE administration. Perfusion of low ET-1 concentrations into the skin induced cutaneous microvasculature vasodilatation in both control and AE-PCOS subjects. The logED50 of the ET-1–%maxCVC dose–response curve was shifted right in lean women with AE-PCOS relative to lean controls (Fig. 1 and Table 3, $P < 0.04$). In contrast, there was no difference in the logED50 of this curve between obese women with and without AE-PCOS (Fig. 1 and Table 3). The logED50 of the ET-1–%maxCVC curve was similar between lean insulin-sensitive AE-PCOS compared to obese,

Table 2. Hormone concentrations before and after 7-day ethinyl oestradiol (EE) administration

		Lean		Obese	
	Hormone	Control	AE-PCOS	Control	AE-PCOS
Pre-EE	$[ET\text{-}1]_S$ (fmol)	0.8 ± 0.4	1.5 ± 0.7*	0.8 ± 0.4	1.5 ± 0.7*
	$[E2]_S$ (pg ml^{-1})	83.7 ± 43.0	103.0 ± 59.9	81.0 ± 37.0	111.7 ± 60.6
	$[P4]_S$ (ng ml^{-1})	1.4 ± 2.9	0.4 (0.2)	1.7 ± 1.7	0.3 ± 0.2
	$[T_{total}]_S$ (ng dl^{-1})	1.8 ± 1.0	2.3 (0.8)	1.3 ± 0.8	2.9 ± 0.9*
	$[T_{free}]_S$ (pg ml^{-1})	1.5 ± 0.9	3.3 ± 1.5*	1.8 ± 0.9	4.7 ± 2.1*
EE	$[ET\text{-}1]_S$ (fmol)	1.4 ± 0.8	0.8 ± 0.2	0.8 ± 0.2	1.2 ± 0.5
	$[E2]_S$ (pg ml^{-1})	76.8 ± 35.2	73.1 ± 32.6	63.6 ± 40.7	79.5 ± 2 7.7
	$[P4]_S$ (ng ml^{-1})	1.3 ± 2.2	0.9 ± 1.1	0.9 ± 1.7	0.1 ± 0.1
	$[T_{total}]_S$ (ng dl^{-1})	1.6 ± 0.8	1.9 ± 0.8	1.1 ± 0.6	2.1 ± 0.8
	$[T_{free}]_S$ (pg ml^{-1})	1.7 ± 0.7	2.1 ± 1.3	1.6 ± 0.9	3.5 ± 1.4*

*Significantly different from control within BMI group. Data are presented as means ± SD. ET-1, endothelin- 1; E2, 17-β-oestradiol; P4, progesterone; T_{total}, total testosterone; T_{free}, free testosterone.

IR AE-PCOS women (Table 3). With ET_BR inhibition, logED50 was increased in all groups with the exception of the control obese women (Table 3, $P = 0.05$). With NO inhibition, logED50 was increased in controls but not in women with AE-PCOS (Table 3). Finally, changes in the logED50 of the ET-1–CVC%max dose–response curve was unaffected by obesity or IR within both the control and the AE-PCOS groups (Fig. 2).

EE administration. Ethinyl oestradiol administration augmented the cutaneous vasodilating response to ET-1 compared to Pre-EE in lean and obese women with AE-PCOS, as indicated by the leftward shift of the logED50 of the ET-1–%maxCVC dose–response curve (Fig. 3 and Table 3, $P = 0.001$). LogED50 was increased with ET_BR inhibition in all groups (Table 3, $P = 0.05$). In contrast, EE had no impact on the ET-1 vasodilatory effect in either lean or obese healthy controls (Fig. 3 and Table 3). As with the Pre-EE, the ET-1-induced vasodilatation during EE administration appears mediated by both NO and ET_BR in lean and obese controls (Table 3). Notably, the response to EE in AE-PCOS subjects suggests that oestrogen can overcome the androgen-induced, endothelium-dependent defect in NO production (Table 3).

AR-dependent inhibition of endothelial ET-1 response

The results described above show that there is a defect in ET-1-induced microvascular dilatation and NO production in women with AE-PCOS. To address whether this phenomenon can be recapitulated at the cellular/molecular level, thereby initiating dissection of molecular mechanisms, confluent EC monolayers were pretreated with the androgen dihydrotesterone (DHT) for 15 h prior to ET-1 stimulation. Figure 4*A* shows that androgen exposure abrogates ET-1-induced EC NO production, as determined by DAF-FM fluorescence. A similar trend was observed for a DHT inhibitory effect on

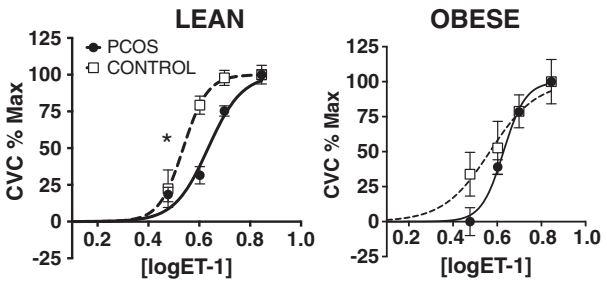

Figure 1. Vasodilatory responses to low dose ET-1: Impact of AE-PCOS
Dose–response curves during cutaneous microdialysis perfusions of low dose endothelin-1 (ET-1) in lean (left) and obese (right) women with polycystic ovary syndrome (PCOS) *versus* controls. Response is shown as percentage maximal cutaneous vasodilatation (%maxCVC). *Significantly different from control within BMI group. Data are presented as means ± SEM.

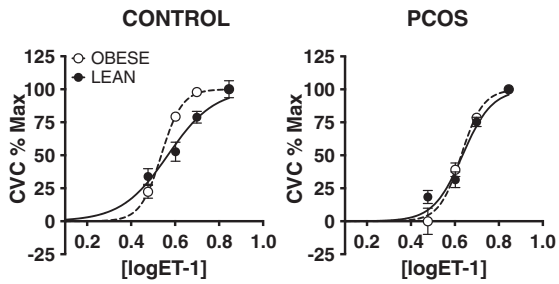

Figure 2. Vasodilatory responses to low dose ET-1: Impact of obesity
Dose–response curves during cutaneous microdialysis perfusions of low dose endothelin-1 (ET-1) in women with polycystic ovary syndrome (PCOS; left) and controls (right), lean *versus* obese. Response is shown as percentage maximal cutaneous vasodilatation (%maxCVC). Data are presented as means ± SEM.

Table 3. Log ED_{50} and Hill slope of cutaneous microdialysis perfusions of low dose endothelin-1 (ET-1) in lean and obese women with and without polycystic ovary syndrome (PCOS, control) during exposure to the blocking agents BQ-788 (ET_BR) and l-N^G-nitroarginine methyl ester (l-NAME, eNOS)

			Lean		Obese	
			Control	AE-PCOS	Control	AE-PCOS
Pre-EE	ET-1	logED50 (nM)	0.49 ± 0.09	0.59 ± 0.08*	0.60 ± 0.13	0.65 ± 0.09
		Hill slope (%CVC_{max}/log[ET])	3.12 ± 4.71	5.29 ± 9.51	3.36 ± 7.70	3.67 ± 3.67
	BQ 788	logED50 (nM)	0.64 ± 0.17§	0.64 ± 0.22§	0.59 ± 0.28	0.70 ± 0.21§
		Hill slope (%CVC_{max}/log[ET])	12.40 ± 10.1	14.61 ± 12.1	7.07 ± 30.81	7.06 ± 23.76
	L-NAME	logED50 (nM)	0.56 ± 0.09§	0.60 ± 0.26	0.67 ± 0.25§	0.56 ± 0.24
		Hill slope (%CVC_{max}/log[ET])	5.88 ± 7.20	6.14 ± 21.80	5.38 ± 15.87	14.39 ± 23.90
EE	ET-1	logED50 (nM)	0.62 ± 0.05	0.29 ± 0.21*,†	0.57 ± 0.16	0.47 ± 0.09*,†
		Hill slope (%CVC_{max}/log[ET])	4.11 ± 5.61	4.62 ± 20.11	4.24 ± 6.60	6.20 ± 8.19
	BQ 788	logED50 (nM)	0.70 ± 0.26§	0.62 ± 0.16§	0.65 ± 0.28§	0.67 ± 0.57§
		Hill slope (%CVC_{max}/log[ET])	6.34 ± 22.72	7.02 ± 18.65	18.96 ± 10.96	15.54 ± 24.42
	L-NAME	logED50 (nM)	0.71 ± 0.18§	0.39 ± 0.77§	0.64 ± 0.28§	0.58 ± 0.52§
		Hill slope (%CVC_{max}/log[ET])	7.18 ± 25.97	2.60 ± 10.06	6.57 ± 11.61	4.44 ± 24.65

*Significantly different from control within BMI group; †significantly different, lean *vs.* obese; §significantly different from ET-1 alone within BMI/PCOS group. Data are presented as means ± SD.

NO induced by the prototypical eNOS agonist VEGF-A, suggesting an androgen-induced signalling defect beyond that observed in response to ET-1. To assess whether this inhibitory effect is AR-dependent, ECs were pretreated with the AR antagonist 2H-flutamide for 30 min prior to DHT addition. Figure 4*B* demonstrates that 2H-flutamide prevents the DHT-induced inhibition of ET-1-stimulated EC NO production. The AR dependence of this inhibitory effect raises a spectrum of mechanistic possibilities (see Discussion).

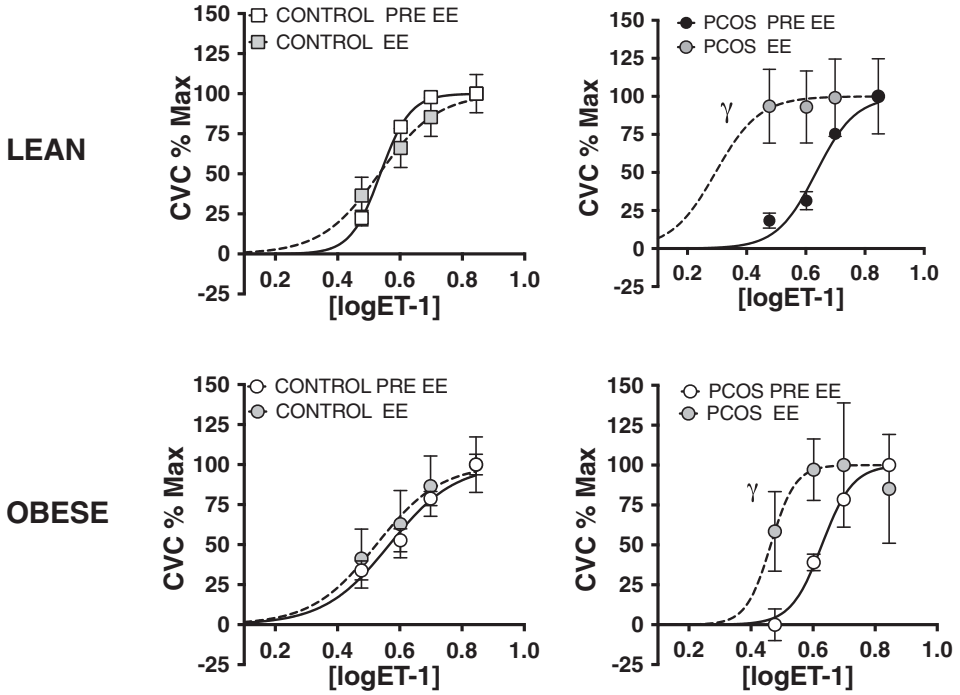

Figure 3. Effects of ethinyl oestradiol treatment on low dose ET-1 induced vasodilation
Dose–response curves during cutaneous microdialysis perfusions of low dose endothelin-1(ET-1) in lean and obese women with and without polycystic ovary syndrome (PCOS, control) before (Pre-EE) and during 7-day ethinyl oestradiol (EE) administration. Response is shown as percentage maximal cutaneous vasodilatation (%maxCVC). γ Significantly different from Pre-EE *versus* EE. Data are presented as means ± SEM.

Discussion

We demonstrated that there is a dose–response relationship between ET-1 perfusion in low concentrations into the cutaneous microvasculature and vasodilatation in women with and without AE-PCOS. Our data extend earlier findings demonstrating ET_BR-mediated vasodilatory actions in the endothelium of the cutaneous circulation predominate over ET_BR-mediated vasoconstrictor actions in vascular smooth muscle in women when applied in low doses. We demonstrated that this ET-1-associated vasodilatation is attenuated in lean, insulin-sensitive women with AE-PCOS compared to obese women with AE-PCOS, suggesting an important role for androgens in the association of AE-PCOS with cardiovascular disease. Further, endothelium-mediated vasodilatation in the skin microvasculature was similar between IR, obese women *vs.* lean women with AE-PCOS (Figs 1and 2). Moreover, while ET-1-induced vasodilatation was attenuated by both NO and ET_BR blockade in lean controls, blockade of NO using L-NAME had no effect on ET-1 responses in women with AE-PCOS prior to EE, indicating ET_BR depends on a different molecular pathway to induce vasodilatation in AE-PCOS. Taken together, our *in vivo* data indicate that: (1) low concentration ET-1 perfusion induces vasodilatation in cutaneous microvasculature of healthy women, through the NO and the ET_BR pathways; (2) low concentration ET-1 perfusion induces vasodilatation in cutaneous microvasculature in insulin-sensitive, lean women with AE-PCOS, but this vasodilatation is attenuated compared to healthy lean women, indicating impaired endothelial functional responses (Fig. 1); (3) ET-1-mediated vasodilatation is similar in lean insulin-sensitive AE-PCOS compared to compared to obese, IR AE-PCOS women, indicating an important role for androgens (Fig. 2); (4) ET-1-induced vasodilatation does not depend on the NO pathway to induce vasodilatation in AE-PCOS; (5) EE administration improves ET-1-induced vasodilatation in AE-PCOS most probably by increasing NO synthesis. Finally, our data show that EE administration has little effect on endothelial function in healthy women.

Healthy endothelial function may be described as a balance of endothelial vasoconstrictor/vasodilator factors. There are sex differences in ET receptor density, and the ratio of receptor subtypes in human vascular tissue. The saphenous veins from men contain greater ET_AR compared to women, and women contain greater endothelial ET_BR receptors relative to men (Ergul *et al.* 1998). In cutaneous microdialysis experiments, an ET_BR antagonist induced vasodilatation in men but vasoconstriction in women (Kellogg *et al.* 2001), suggesting that ET_BRs in men are expressed primarily on vascular smooth muscle and mediate vasoconstriction, while these same receptors in women are primarily expressed on the endothelium and mediate vasodilatation. Stimulating the peripheral microvessels with low, physiological doses of ET-1 induces vasodilatation in women, which is consistent with the finding that at low doses of ET-1 small numbers of the ET_BR receptors are occupied (Ergul *et al.* 1998). At higher perfusion doses (>1 μM), the ET_BRs on the endothelium are saturated, so ET-1 effects are also seen

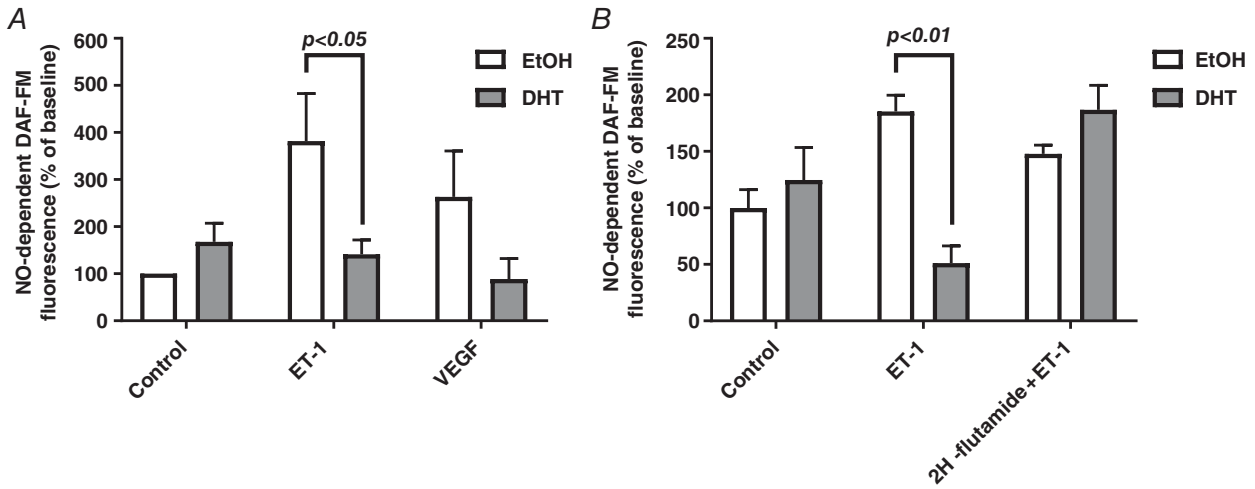

Figure 4. AR-dependent effects of DHT on ET-1- and VEGF-induced EC NO production
NO production was measured by fluorescence of DAF-FM diacetate in BAECs pretreated with DHT (70 nM) or solvent control overnight and stimulated with ET-1 (100 nM) or VEGF (10 ng ml^{-1}) for 15 min (*A*), in the presence or absence of the AR antagonist 2H-flutamide (10 μM; *B*). DAF-FM fluorescence in cells treated with agonists in the presence of L-NAME (1 mM) was subtracted from DAF-FM fluorescence in the absence of L-NAME to determine the NO-dependent signal. Data are presented as means ± SEM of four independent experiments, each with a minimum of triplicate wells per treatment group.

in the vascular smooth muscle, where vasoconstriction is induced, even in women. Based on our data, this general system appears to hold in AE-PCOS, but is affected by the hyperandrogenism, and is independent of both IR and adiposity.

Testosterone is integral to the development of endothelial dysfunction and hypertension in IR rats (Vasudevan *et al.* 2005; Vasudevan *et al.* 2006), and it is likely that differences in $[\text{ET-1}]_S$ between our AE-PCOS and control groups is related to $[T_{free}]_S$. Serum concentrations of ET-1 were higher in both lean and obese women with AE-PCOS compared to healthy women, indicating endothelial injury. Gonadotropin releasing hormone suppression (and associated oestradiol suppression) increases $[\text{ET-1}]_S$ in healthy women, but causes decreases in $[\text{ET-1}]_S$ in women with AE-PCOS, corresponding to $[T_{free}]_S$ suppression (Wenner *et al.* 2013). In contrast, short-term testosterone administration increases $[\text{ET-1}]_S$ (McCrohon *et al.* 1999; Paradisi *et al.* 2001; Bajuk Studen *et al.* 2011; Wenner *et al.* 2013).

Nitric oxide mediates the cholinergic vasodilatory response in the skin in healthy women (Holowatz *et al.* 2005; Bruning *et al.* 2012). Thus, we were surprised to see little effect of L-NAME in the skin in women with AE-PCOS before the EE treatment (Table 3). This system has not been studied before in AE-PCOS, and neither has the impact of androgens on this vasodilatory system in women in general, but these findings demonstrate that the endothelial dysfunction in AE-PCOS is mediated through an androgen effect on the NO pathway. Androgen exposure can result in impaired agonist-triggered endothelial NO release in women, and AE-PCOS is associated with inflammation and inflammatory cytokines, oxidative stress and NF-κB activation (Diamanti-Kandarakis, 2008). The combination of high oxidative stress and poor endothelial dysfunction may lead to low levels of NO (Yavuz Taslipinar *et al.* 2014; Krishna *et al.* 2017), as a result of reduced iNOS/eNOS expression concomitant with other factors of inflammation (Krishna *et al.* 2017). These findings are of special interest here because earlier studies have shown similar outcomes that were independent of obesity, blood lipids and IR (Yavuz Taslipinar *et al.* 2014). We propose that the relative insensitivity to NO blocking in the skin in women with AE-PCOS is likely to be associated with low levels of NO production. We now propose that the androgen dominant hormonal milieu of AE-PCOS results in low NO production, indicating that ET-1-induced vasodilatation occurs through pathways other than that of NO. This argument is strengthened by what seems to be an increase in ET-1-mediated vasodilatation with EE administration and the likelihood of increased NO synthesis.

The *in vitro* endothelial cell data are consistent with the cutaneous vasodilatation results, and begin to approach a mechanistic dissection of this phenomenon. There is a clear androgen-induced EC signalling defect observed at the level of NO detection, which is not specific to an effect on the ET_BR, as there is a similar trend towards an impaired response to VEGF. This suggests a global endothelial dysfunction state, which has been suggested for women with PCOS and correlated to the early development of cardiovascular disease. The androgen-induced reduction in endothelial NO production is consistent with the lack of an L-NAME effect observed in our AE-PCOS subjects, that is, the ET-1-induced vasodilatation in these subjects, reduced compared to controls, was not NO dependent.

Our EC results present a clear and easily interpretable assay with which to dissect molecular mechanisms of this inhibitory DHT effect. Although the epidemiological data regarding testosterone and cardiovascular disease in men have been divergent (Basaria *et al.* 2010; Jones *et al.* 2011), and thus to date inconclusive, it is likely that differential vascular androgen receptor responses occur in male and female endothelial (and smooth muscle) cells. The *in vitro* results presented here were obtained with bovine ECs of unknown sex origin. However, as opposed to simply studying whether androgens alone drive or repress NO production, our assay focused on the influences of AR engagement on NO-inducing agonists, namely ET-1 and VEGF. The AR antagonist data confirm that the androgen-induced inhibitory effect is AR mediated. This is important because, although steroid hormones preferentially engage their specific receptor, there is promiscuity within the steroid hormone ligand-receptor family. Furthermore, other molecules, including channels such as the transient receptor potential cation channel subfamily M member 8 (TRPM8) (Asuthkar *et al.* 2015), and G-coupled protein receptors such as GPRC6A (Pi *et al.* 2015) can be bound, modified and activated by androgens in an AR-independent fashion. Our data conclusively demonstrate that AR function is required for the inhibitory effect.

The simplest explanation of a possible AR-mediated NO suppressive effect is DHT induction of reactive oxygen species (ROS). Testosterone has been shown to induce ROS formation by both AR-dependent (Montezano *et al.* 2015) and -independent (Ko *et al.* 2014) mechanisms. This includes NADPH-oxidase-dependent ROS generation in ECs (Costa *et al.* 2015). Androgens have been shown to both increase NADPH oxidase subunits levels, including Nox1, Nox4 and p47phox (Montezano *et al.* 2015), and to promote posttranslational modification of Nox subunits (Costa *et al.* 2015). Superoxides, through interaction with NO, can reduce NO bioavailability. Thus, it is possible that ligand-induced eNOS activation proceeds normally but that NO is rapidly consumed by intracellular superoxide reactivity. Other possibilities include that DHT is altering membrane receptor localization and/or function. If this is the case, multiple classes of receptors must be affected, including G protein-coupled receptors

(ET_BR) and tyrosine kinase receptors (VEGFR2). With this strong inhibitory signal in our assay, the precise level and molecular nature of this AR-dependent inhibitory effect will be defined in future studies. Furthermore, the ∼15 h pretreatment with DHT raises the possibility that this inhibitory effect could be the result of either rapid, membrane-initiated, non-genomic signalling through the AR, or genomic consequences of AR engagement, most notably transcriptional influences on genes critically involved as either activators or repressors of the eNOS-mediated NO production pathway. Time course and membrane-impermeant hormone studies are ongoing to distinguish these mechanisms.

Endothelin-1-mediated vasodilator tone is diminished in women with AE-PCOS, demonstrating microvascular endothelial dysfunction. Cardiovascular disease markers are common in women with AE-PCOS, and AE-PCOS is fraught with symptoms that impact on quality of life: infertility, hirsutism, seborrhoea, acne and androgenic alopecia (Azziz *et al.* 2009). Metabolically, AE-PCOS is associated with increased fat storage, android type obesity, dyslipidaemia, IR and excess insulin production (Gambineri *et al.* 2002; Azziz *et al.* 2009). One-third of women with AE-PCOS develop metabolic syndrome by 49 years; many develop this syndrome before they reach 40 years. This is in stark contrast to the 6.7% prevalence of metabolic syndrome in US women between ages 20 and 30 years, and the 15% prevalence in women between ages 30 and 40 years (Ford *et al.* 2002). At least 50–60% of women with AE-PCOS are obese (Gambineri *et al.* 2002), compared to ∼30% in the general female population (Flegal *et al.* 2002). While these data indicate that androgens are the primary driver for endothelial dysfunction in AE-PCOS, hyperinsulinaemia exacerbates the problem of hyperandrogenism by inducing ovarian and adrenal androgen production. Our present investigation adds to and supports our earlier studies in women with AE-PCOS in which we have indicated lower microvascular responsiveness in these women relative to healthy controls (Wenner *et al.* 2011*a*, 2013). In addition, these findings are consistent with those from animal models with androgen-induced endothelial dysfunction together with insulin sensitivity in normal weight animals (Hurliman *et al.* 2015).

Limitations

In order to avoid saturating the ET_BR on the endothelium we used very low doses of ET-1 in this study. This led to high errors for some of the skin blood flow data (in the Hill slope), making them difficult to interpret at times (see Table 3). However, we feel confident in our findings because they are consistent with the findings in our molecular studies.

Conclusions

Ours is the first study to examine the androgen effects on mechanisms associated with endothelial dysfunction in women with AE-PCOS while controlling for the other important variables that may influence these outcomes. We demonstrated that the hyperandrogenic milieu in women with AE-PCOS is the primary factor associated with endothelial dysfunction, and also demonstrated that the ET_BR plays an important role in this dysfunction. Further our data strongly indicate that the androgen-dominant hormonal milieu in women with AE-PCOS suppresses NO production, also leading to endothelial dysfunction. Our physiological results were recapitulated at the cellular level, with complete inhibition of ET-1-induced NO production in androgen-pretreated ECs. Future studies will examine the impact of compromised inflammatory systems in these women, determine the androgen contribution to changes in this system, and dissect the molecular mechanism(s) responsible for the impaired responses to eNOS agonists in women with AE-PCOS.

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Additional information

Competing interests

None declared.

Author contributions

C.W.U. ran the skin blood flow experiments and analysed the data, and collaborated in writing the manuscript; J.R.B analysed the data from the molecular experiments, and collaborated in writing the manuscript; T.W. ran the molecular experiments and analysed the data; F.S. ran the molecular experiments; H.S.T. assisted with the design of the project and screened and supervised the subjects on medication; C.L. assisted with all human experiments, conducted the hormone assays and analysed the hormone data; N.S.S. supervised the project, assisted in data collection, aided in interpreting results, and collaborated in writing in the manuscript; C.W.U., N.S.S. and J.R.B devised the project. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Translational perspective

Polycystic ovary syndrome (PCOS) affects approximately 1 in 10 women worldwide and is a leading cause of infertility. While PCOS is often thought of as a reproductive disorder, PCOS is associated with a number of co-morbidities, including insulin resistance, obesity, high cholesterol and lipids, high blood pressure and cardiovascular disease. Polycystic ovary syndrome is also associated with elevations in the male hormone testosterone. In this study, we tested the hypothesis that elevations in testosterone exert deleterious effects on endothelial function independent of the metabolic comorbidities of PCOS. We also hypothesized that oestradiol supplementation would reverse these cardiovascular effects and improve endothelial function in women with PCOS. Our study confirmed our hypothesis, indicating that testosterone plays an important role in cardiovascular disease even in lean, insulin-sensitive women with PCOS. Further, low levels of oestradiol administration were able to improve endothelial function in the peripheral blood microvasculature in both lean and obese women with PCOS. Thus, our data indicate that testosterone is associated with increased cardiovascular risk in women with PCOS *independently* of metabolic risk factors, and that oestradiol is a viable intervention to improve endothelial function in young women with PCOS. We also demonstrated that the effects of testosterone on endothelial function were dependent on the endothelin B receptor, and future research should focus on molecular pathways for these androgen effects on endothelial cells in PCOS.