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Abstract

Hypothesis: There are fetal sex-associated differences in the circulating maternal renin–angiotensin system (RAS) in early pregnancy.

Methods: Plasma prorenin, angiotensin (Ang) II, Ang I-7 and angiotensin-converting enzyme (ACE) concentrations were measured at 15 weeks' gestation in 131 women with uncomplicated pregnancies from the Adelaide SCOPE cohort. Uterine and umbilical artery Doppler sonography was performed at 20 weeks' gestation.

Results: At 15 weeks, women bearing female fetuses had higher maternal Ang II concentrations ($p = 0.017$) and lower Ang I-7 to Ang II ratios ($p = 0.016$) than women bearing males. In women with male fetuses, Ang II positively correlated with birth weight ($p = 0.028$) and prorenin negatively correlated with placental weight ($p = 0.014$). Female fetuses had higher umbilical artery resistance indices ($p = 0.019$) that were related to maternal prorenin concentrations ($p = 0.007$).

Conclusions: In early human pregnancy, the maternal RAS is influenced by fetal sex. The lower Ang I-7 to Ang II ratios in women with female fetuses may contribute to the lower maternal peripheral microvascular flow as described previously and the lack of any positive effect of Ang II on fetal growth, as seen in women with male fetuses.

Keywords

Pregnancy, sexual dimorphism, prorenin, angiotensin-converting enzyme, angiotensin peptides

Introduction

Pregnancy places increased stress on the maternal cardiovascular system. The renin–angiotensin aldosterone system (RAAS) is a hormone cascade responsible for blood pressure homeostasis and is upregulated during pregnancy.^{1–3} A significant increase in plasma volume is essential for a successful pregnancy; these changes in plasma volume are mediated in part by the actions of angiotensin (Ang) II on the adrenal cortex, which releases aldosterone and stimulates fluid retention.⁴

Ang II has vasopressor effects, increasing systemic vascular resistance through central and peripheral actions. The RAAS is activated in early pregnancy to maintain blood pressure as maternal peripheral blood flow increases, particularly in the skin,⁵ kidneys⁶ and uterus.⁷ A rise in glomerular filtration rate (GFR)⁸ and an increase in progesterone levels⁹ occur early in pregnancy and would promote salt excretion if not offset by an activated RAAS. As well, sodium retention has to occur to allow for the

increase in maternal blood volume and to meet the demands of the growing fetus.

In pregnant women, plasma levels of active renin, and its precursor prorenin, are increased. Prorenin levels increase to 100 times those of active renin, while in non-pregnant individuals they are only 10 times greater.^{10–12} Under physiological conditions, 2% of this prorenin will spontaneously activate,¹³ thereby adding to the circulating renin enzyme activity. Recently, we showed that decidual

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explants collected at term from women carrying a female fetus secrete more prorenin *ex vivo* than explants collected at term from women with a male fetus.¹⁴ Furthermore, these sex-specific effects on decidual prorenin secretion were still apparent when decidual explants were incubated for 48 hours *ex vivo*.

The activity of the RAAS is strongly influenced by the sex steroids, oestrogen and progesterone.^{15–17} Most significant is the effect of oestrogen on hepatic production of angiotensinogen (AGT).¹⁸ Renin cleaves AGT to form Ang I; a reaction rate limited by AGT levels. Ang I is cleaved by angiotensin-converting enzyme (ACE) to produce Ang II. Angiotensin-converting enzyme 2 (ACE2) is the predominant enzyme for producing the heptapeptide Ang 1-7, with this reaction 500 times faster when using Ang II as the substrate rather than being generated from Ang I.¹⁹ Renin and prorenin are also influenced by sex, being lower in the circulation in women than in men, possibly because the influence of oestrogens on AGT generates more Ang II leading to negative feedback on renin release.^{20,21} Conversely, Broughton-Pipkin *et al.*,²² who examined the renin and angiotensin levels in children less than 8 years of age, have found that plasma renin activity and Ang II levels are lower in boys than girls.

Ang peptides have been measured in pregnancy and both plasma Ang II and Ang (1-7) are increased.^{23,24} Urinary Ang 1-7 also increases throughout gestation.²⁵ Ang II has not been measured in early gestation at the same time as Ang 1-7. Ang 1-7 has been shown to be a vasodilator by inducing endothelial nitric oxide (NO) production,²⁶ kinins²⁷ and prostaglandins²⁸ so the balance between the levels of Ang II and Ang 1-7 may be important in regulating maternal vascular tone. Interestingly, in preeclampsia and gestational diabetes Ang (1-7) levels are low in late gestation.^{29,30} In order to fully elucidate the functional role of these peptides in pregnancy and the use of Ang 1-7 and/or Ang II as potential biomarkers for pregnancy pathologies such as preeclampsia and gestational hypertension in which the RAAS has been implicated,³¹ we first needed to establish the normal levels of these peptides and other components of the RAAS in early gestation in women with uncomplicated pregnancies.

We therefore tested the following hypotheses: first, that a factor associated with fetal sex alters the balance between the Ang II and Ang 1-7 axes of the maternal RAS; second, these sex-based differences in angiotensin peptides are due to differences in the upstream components of the RAS, *i.e.* prorenin and ACE; third, since the circulating RAAS is a key regulator of cardiovascular and renal function and Ang II is a pro-inflammatory peptide,^{32,33} these differences in circulating RAAS components would affect other measures of maternal cardiovascular and renal health as well as a marker of inflammation (C-reactive protein; CRP). Finally, we postulated that differences in Ang peptides, and therefore differences in the vasodilator/vasoconstrictor balance,

would alter uterine artery and umbilical artery resistance indices as well as fetal growth.

Materials and methods

Study design

The current study is a nested case-control study within the Adelaide Screening for Pregnancy Endpoints (SCOPE) cohort. Women attending the Lyell McEwin Hospital (South Australia, Australia) were recruited, after giving informed written consent, if they were nulliparous with a singleton pregnancy, less than 15 weeks' gestation and had fewer than three previous terminations of pregnancy or miscarriages. Samples collected at 15 weeks' gestation were selected if women had no pregnancy-associated complications ($n = 131$), that is, if they remained normotensive (<140 and/or <90 mmHg prior to labour), showed no proteinuria, delivered a live born baby who was normally grown after 37 weeks' gestation and had no other sign of pregnancy complications. All the women included in the current study self-reported as being Caucasian, except for one who was listed as a Maori/Cook Islander. Ethics approval for this work was given by the Central Northern Adelaide Health Service Ethics of Human Research Committee (study number: REC 1714/5/2008).

Sample collection

Non-fasting blood was collected into ethylenediaminetetraacetic acid (EDTA) vacutainers at 15 weeks' gestation by venepuncture from patients who had been either sitting or supine for 10 minutes (min). Blood pressure and other clinical measurements were also recorded at this time. Mid-stream urine was collected into 50 ml pots. All samples were placed on ice before processing and storage at -80°C within 30 min of collection. Doppler sonography was conducted at 20 weeks' gestation on the umbilical and uterine arteries to measure blood flow resistance. Birth and placental weights were measured following delivery. Maternal daily cigarette consumption from before and during pregnancy was also recorded.

Laboratory measurements

Plasma Ang II and Ang 1-7 were measured at ProSearch International Australia Pty. Ltd. (Malvern, Victoria, Australia) using a direct radioimmunoassay employing delayed tracer addition. Ang II assay sensitivity was 4 pmol/l; with intra- and inter-assay coefficients of variation of 6.4% and 12%, respectively. Cross-reactivity to Ang I, Ang 1-7 and all other pertinent hormones is 0.52%, 0.01% and <0.1%, respectively. Cross-reactivity to Ang III and Ang IV are 98% and 100%, respectively, as these peptides have the same c-terminal as Ang II. Quality controls for

Ang II measured 35.9, 37.5 and 33.8 pmol/l. Ang 1-7 assay sensitivity was 13 pmol/l; with intra- and inter-assay coefficients of variation of 4.5% and 10%, respectively. Cross reactivity to Ang I, Ang II, Ang III and Ang IV were 0.11%, 0.04%, 0.53% and 0.25%, respectively. Quality controls for Ang 1-7 measured 135.8, 158.2 and 149.2 pmol/l. The Ang 1-7 to Ang II ratios were derived from the plasma concentrations of these peptides.

Maternal plasma concentrations of ACE (Duoset, R&D Systems, MN, USA) and prorenin (Molecular Innovations, MI, USA) were measured using commercially available enzyme-linked immunosorbent assay kits and conducted according to manufacturer's instructions.

Biological analytes were measured by SA Pathology at the Institute of Medical and Veterinary Science (South Australia, Australia). Plasma and urinary electrolytes were measured using ion-selective electrodes, while creatinine was measured using the Jaffé method and CRP determined with an immuno-turbidimetric assay. All assays were read on an Olympus AU5400 Chemistry-Immuno Analyzer.

Data analysis

The rate pressure product, which is an indirect measure of cardiac work, was calculated by multiplying heart rate and systolic blood pressure. Plasma creatinine was used as a surrogate measure of GFR because equations that derive GFR from the plasma creatinine cannot be used in pregnancy because of the physiological changes in GFR and the progressive changes in body weight.³⁴ Urinary protein to creatinine, urinary albumin to creatinine and urinary sodium to potassium ratios were determined and the fractional excretion of sodium calculated from the formula:

$$\text{FENa (\%)} = (\text{UNa} \times (\text{PCr}/1000))/(\text{PNa} \times \text{UCr}) * 100$$

Where UNa and PNa are urinary and plasma sodium levels (mmol/l), respectively, and UCr and PCr are urinary (mmol/l) and plasma creatinine levels ($\mu\text{mol/l}$), respectively.

Values are expressed as medians and interquartile ranges, unless otherwise stated. Statistical significance was deemed as $p < 0.05$. Mann-Whitney *U* tests were used to determine fetal sex-dependent differences (male fetuses: $n = 68$, female

Table 1. Measures of maternal health in women who had a normal pregnancy outcome and either a singleton female fetus or a singleton male fetus.

Maternal characteristics	Female fetus		Male fetus	
	Median (IQR)	<i>n</i>	Median (IQR)	<i>n</i>
Age	23 (20–27)	63	23 (19–27)	68
Cigarettes/day	0 (0–6)	63	0 (0–15)	68
Height (cm)	165 (158–169)	63	164 (159–168)	68
Weight (kg)	69 (59–96)	63	69 (57–88)	68
Cardiovascular measurements				
Systolic BP (mmHg)	108 (102–116)	63	108 (100–115)	68
Diastolic BP (mmHg)	64 (58–70)	63	60 (60–68)	68
Heart rate (beats/min)	82 (75–87)	63	84 (78–88)	68
Rate pressure product (bpm*mmHg)	8820 (8000–9800)	63	8754 (8144–9723)	68
Plasma and urinary analytes				
Plasma CRP (mg/l)	6 (3–11)	62	4 (2–8)	67
Plasma sodium (mmol/l)	138 (136–144)	56	138 (136–143)	58
Plasma urea (mmol/l)	2.7 (2.4–3.0)	57	2.7 (2.1–3.1)	60
Plasma creatinine ($\mu\text{mol/l}$)	46 (42–51)	63	46 (41–52)	67
Urinary protein (g/l)	0.07 (0.05–0.12)	63	0.07 (0.05–0.16)	67
FENa (%)	0.5 (0.3–0.7)	54	0.4 (0.3–0.6)	57
Urinary sodium:potassium	2.5 (1.5–3.1)	60	2.0 (1.2–2.8)	67
Urinary albumin:creatinine (mg/mmol)	0.5 (0.4–0.6)	59	0.5 (0.4–0.8)	64
Urinary protein:creatinine (g/mmol)	0.011 (0.009–0.014)	63	0.010 (0.008–0.015)	67
Obstetric data				
Birth weight (g)	3460 (3290–3830)	63	3,483 (3271–3765)	68
Placental weight (g)	600 (500–705)	60	596 (500–700)	63
Umbilical artery RI	0.76 (0.73–0.79) ^a	61	0.74 (0.71–0.78)	68
Uterine artery RI	0.58 (0.53–0.62)	61	0.60 (0.54–0.63)	68

All data were collected at 15 weeks' gestation, except the obstetric data, which were measured at 20 weeks' gestation by Doppler sonography, and presented as median (interquartile range); birth and placental weights were collected at term. IQR: interquartile range; BP: blood pressure; CRP: C-reactive protein; FENa: fractional excretion of sodium; RI: resistance index; ^a $p = 0.019$ using Mann-Whitney *U* tests.

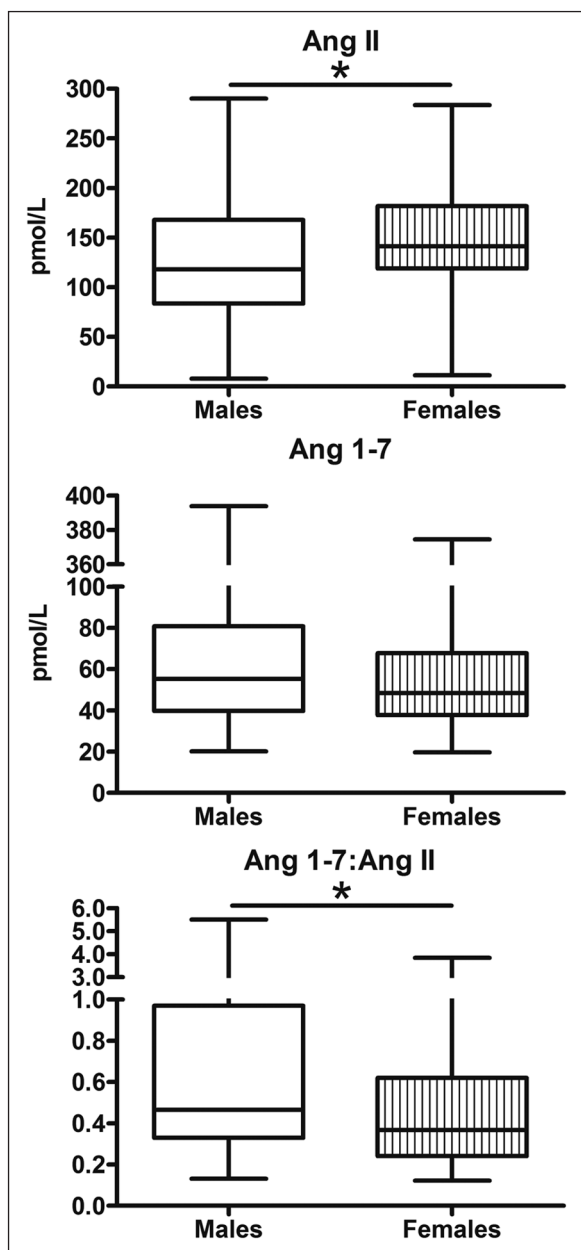


Figure 1. Maternal angiotensin peptides were influenced by the sex of the fetus. Maternal plasma was collected at 15 weeks' gestation and angiotensin (Ang) II and Ang 1-7 measured and the ratio between them calculated. Mann-Whitney U tests used; * $p < 0.02$.

fetuses: $n = 63$). Pairwise Spearman correlations were used to identify significant relationships between RAS variables thought to interact on a physiological basis. Data were analysed using Stata/IC 11.0 (StataCorp LP, TX, USA) and scatter plots generated with this software. GraphPad Prism 5.0 was used to generate box and whisker plots.

Results

The only difference in maternal physiological variables, measured at 15 weeks' gestation, between women who

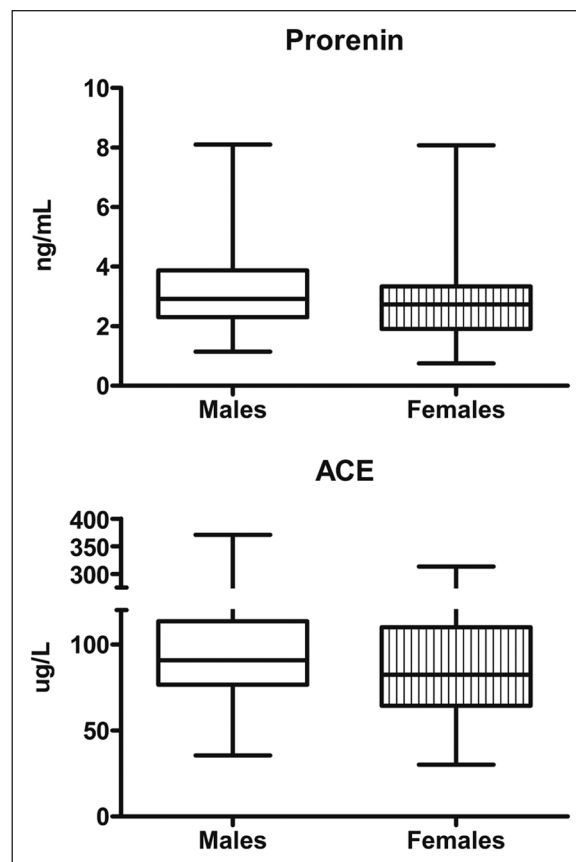


Figure 2. Maternal prorenin and angiotensin-converting enzyme (ACE) were not influenced by fetal sex. Maternal plasma was collected at 15 weeks' gestation and prorenin and ACE measured.

subsequently delivered a female baby compared with those who had a male baby was higher umbilical artery resistance indices at 20 weeks' gestation in women who carried female fetuses (Table 1).

Plasma Ang II levels were higher in women with female fetuses compared with women carrying a male fetus ($n = 68$, $p = 0.017$), but Ang 1-7 levels were the same irrespective of fetal sex (Figure 1). Therefore, Ang 1-7 to Ang II ratios were lower in women carrying female fetuses compared to males ($p = 0.016$, Figure 1). The total concentration of Ang II plus Ang 1-7 was similar in both groups of women, as were plasma ACE concentrations and prorenin concentrations (Figure 2). At 15 weeks' gestation, maternal heart rate, blood pressure, rate pressure product, plasma urea, plasma sodium, plasma creatinine, urinary albumin to creatinine ratio, urinary protein to creatinine ratio and the urinary sodium to potassium ratio were not affected by fetal sex (Table 1).

CRP concentrations were inversely related to maternal prorenin concentrations (Figure 3), as was placental weight if the fetus was male (Table 2). In women with female fetuses, maternal prorenin concentrations at 15 weeks'

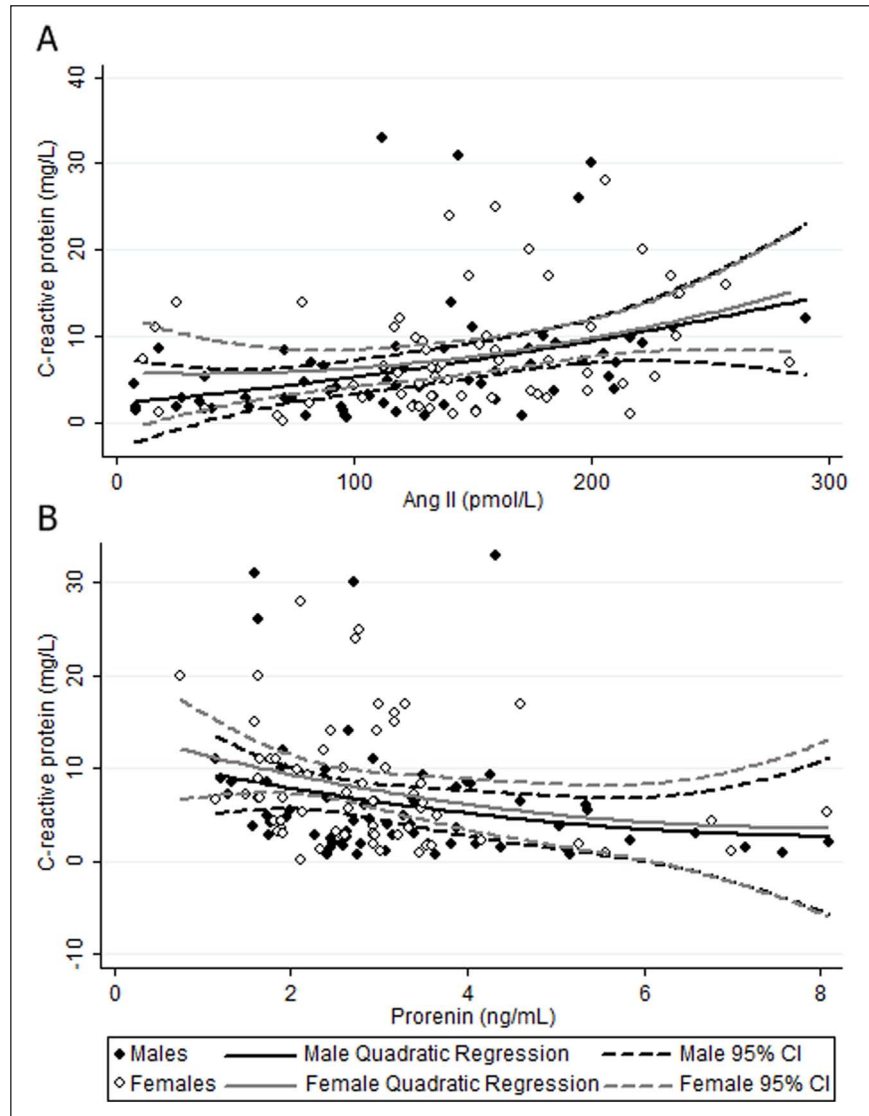


Figure 3. The effect of fetal sex on relationships between the maternal RAS and CRP. (A) There was a positive relationship between maternal plasma Ang II and CRP ($p < 0.01$, $R^2 = 0.12$); whilst (B) maternal circulating prorenin levels showed a negative relationship with plasma CRP ($p < 0.02$, $R^2 = 0.07$) irrespective of fetal sex. All data were obtained from women at 15 weeks' gestation. RAS: renin-angiotensin system; CRP: C-reactive protein; Ang: angiotensin.

gestation positively correlated with umbilical artery resistance indices at 20 weeks' gestation.

In contrast to the negative relationships between maternal prorenin concentrations and CRP concentrations in women with either a male or female fetus, there were positive relationships between Ang II concentrations and CRP (Figure 3). Significantly, there was a sexually determined difference in the relationships between Ang II concentrations at 15 weeks' gestation and birth weight. There was a positive relationship between Ang II concentrations at 15 weeks' gestation and male birth weights that was not seen if the fetus was female (Table 2). There was also a positive correlation in women carrying male fetuses, between Ang 1-7 and maternal heart rate and plasma sodium concentrations.

Only one relationship between the limited measures of renal health that we could obtain and the maternal RAS was found. In women carrying female fetuses, plasma creatinine (a surrogate measure of GFR) was positively related to plasma ACE concentrations (Table 2).

Discussion

The observations that Ang II concentrations are higher if the fetus is female and Ang 1-7 to Ang II ratios lower support our hypothesis that the balance between these RAS peptides is influenced by the sex of the fetus. Our second hypothesis was that any differences in the Ang II and Ang 1-7 axes of the RAS were associated with differences in

Table 2. The effect of fetal sex on relationships between the maternal RAS and estimates of maternal health, including cardiovascular health, laboratory measurements and obstetric data.

Maternal characteristics		Female			Male		
		<i>r</i>	<i>p</i>	<i>n</i>	<i>r</i>	<i>p</i>	<i>n</i>
Cardiovascular							
Heart rate	Ang 1-7	0.106	0.409	63	0.315	0.009	68
Plasma analytes							
Plasma sodium	Ang 1-7	0.090	0.512	56	0.412	0.001	58
Plasma urea	Prorenin	0.277	0.037	57	-0.104	0.430	60
Plasma creatinine	ACE	0.286	0.023	63	0.019	0.877	67
Obstetric data							
Umbilical artery RI	Prorenin	0.343	0.007	61	-0.011	0.927	68
Birth weight	Ang II	-0.121	0.348	62	0.267	0.028	68
Placental weight	Prorenin	0.183	0.161	60	-0.308	0.014	63

All data were collected at 15 weeks' gestation, except the obstetric data, which were measured at 20 weeks' gestation by Doppler sonography; birth and placental weights were collected at term. Pairwise Spearman correlations were used to identify relationships of interest with statistical significance set at 5%. RAS: renin-angiotensin system; Ang: angiotensin; ACE: angiotensin-converting enzyme; RI: resistance index.

maternal prorenin or ACE levels. Since there were no differences in prorenin or ACE levels between the two groups, the higher Ang II concentrations in women with a female fetus did not depend on parallel differences in those factors that control its production, which we measured. There was also no difference between the two groups of women in the total concentration of Ang peptides measured. Taking these two observations into account, the sex-specific differences in maternal Ang II concentrations and in the Ang 1-7 to Ang II ratios in these women could be due to a greater rate of conversion of Ang II to Ang 1-7 in women carrying a male fetus. ACE2, which converts Ang II to Ang 1-7, is found in abundance in the syncytiotrophoblast of both the early- and late-gestation placenta,³⁵⁻³⁸ where it could convert maternal Ang II, perfusing the intervillous space to Ang 1-7. Thus the sex difference in the Ang 1-7 to Ang II ratio could be conferred by exposure of maternal Ang II to male syncytiotrophoblast.

Levels of Ang II and Ang 1-7 were measured in blood collected in EDTA tubes. This was because they were collected for a multicentre study looking for biomarkers of pregnancy outcome. At the time of collection, peptides of the RAS were not considered and so tubes containing protease inhibitors were not made available to the clinic. There may have been some post-collection activity of proteases not sensitive to EDTA. It is unlikely that either ACE or ACE2 were active post-collection since both are inhibited by EDTA³⁹ and plasma ACE2 activity is blocked by an endogenous inhibitor.⁴⁰ Be that as it may, this does not account for the striking sexual dimorphism in the Ang 1-7 to Ang II ratios. The greater amount of Ang II in women carrying female fetuses and the lower Ang 1-7 to Ang II ratios are indicative of a lesser rate of conversion of Ang II to Ang 1-7 in these women. Adult males have higher circulating ACE2 activity than that of adult females⁴¹ and ACE2 activity is

higher in the kidney of male mice due to an increased enzyme velocity.⁴² We suggest that if ACE2 in the syncytiotrophoblast³⁵⁻³⁸ of women carrying male fetuses also exhibits the same higher enzyme velocity, Ang II would be more rapidly converted to Ang 1-7 by the male placenta.

Our third hypothesis was that differences in circulating RAS components would affect other measures of maternal cardiovascular and renal health, as well as a marker of inflammation (CRP; Table 2). Overall there were no sex-specific differences in maternal cardiovascular function or in indices of renal health at 15 weeks' gestation. Although several sex-specific relationships between RAS components and measures of cardiovascular and renal health were found (Table 2), the sex-specific mechanisms behind these relationships are unclear. While these are only associations and require experimental validation, they may imply sex differences later in gestation in women who develop gestational hypertension or preeclampsia.

Stark et al.⁴³ found that near term, women carrying a female fetus had lower peripheral microvascular skin blood flow than women carrying a male fetus. We think that the higher Ang II concentrations in women carrying a female fetus and the consequent lower Ang 1-7 to Ang II ratios could explain this difference in maternal skin blood flow. Ang II is both directly and indirectly a potent vasoconstrictor⁴⁴ and Ang 1-7 a vasodilator,⁴⁵ so the lower Ang 1-7 to Ang II ratios in women carrying a female fetus could result in increased vascular tone. The study by Stark et al.⁴³ also showed a greater increase in maternal peripheral microvascular flow in response to corticotrophin-releasing hormone (CRH) in women carrying male fetuses. This might also be explained by our finding that Ang 1-7 to Ang II ratios were higher in women carrying male fetuses because both Ang 1-7 and CRH increase endothelial NO production.²⁶

If, as postulated above, ACE2 activity is greater in the male fetus and in its placenta than the female, then this could also explain the lower umbilical artery resistance indices of male fetuses compared with female fetuses, since ACE2 is also found in abundance in the fetal endothelium of the villous placenta.^{35,38}

In both groups of pregnant women, Ang II and CRP concentrations were directly related. CRP is a non-specific marker of inflammation. Ang II is commonly regarded as a pro-inflammatory peptide which has been shown to stimulate CRP synthesis in vascular endothelial cells,³² as well as macrophages,³³ and blockade of the Ang II-AT₁ receptor is associated with decreased plasma CRP concentrations.⁴⁶ CRP is synthesised by the liver in response to factors released from adipocytes and is known to be associated with obesity.⁴⁷ We cannot explain the negative correlation seen in both groups of women between maternal prorenin and CRP. Although it may occur indirectly through negative feedback effect of Ang II on renal prorenin production, we found no evidence for a negative relationship between prorenin and Ang II in maternal plasma in this study (data not shown).

Finally, we postulated that differences in Ang peptides, and therefore differences in the vasodilator/vasoconstrictor balance, would be associated with altered uterine artery and umbilical artery resistance indices as well as fetal growth. Here we have shown that there is a positive relationship between maternal Ang II levels and birth weight in women carrying male fetuses but neither Ang peptide was related to uterine or umbilical artery resistance indices in this control population. In contrast, a positive correlation between maternal prorenin and umbilical artery resistance indices of female fetuses and a negative correlation between maternal prorenin and placental weight of male fetuses was observed, suggesting that prorenin within the utero-placental circulation has adverse effects on fetal health and well-being.

In conclusion, in healthy pregnancies with normal outcomes, maternal plasma Ang II concentrations were higher and Ang 1-7 to Ang II ratios were lower at 15 weeks' gestation if women were carrying a female fetus. This could account for the higher maternal peripheral vascular tone seen at term in pregnancies carrying female fetuses compared to those with males.⁴³ There is no evidence that these differences are due to higher concentrations of prorenin or ACE. It is possible that the rate of conversion of Ang II to Ang 1-7 by ACE2 is slower in women bearing female fetuses.

This work shows for the first time that early in normal human pregnancy the concentration of two biologically significant angiotensin peptides in the maternal circulation is influenced by fetal sex. The higher Ang 1-7 to Ang II ratios could explain findings by others that maternal microvascular skin flow is higher if a woman is carrying a male fetus, as Ang 1-7 is a vasodilator and Ang II is a vasoconstrictor. The higher umbilico-placental vascular resistance of female fetuses and the negative impact of high maternal prorenin

levels on placental weight if the fetus is male further suggest that the intrauterine RAS may influence placental development and function.

This study also highlights the need to take into account fetal sex when studying the role of the maternal circulating RAS in pregnancy.

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Conflict of interest

None declared.

Authors' contributions

Sykes: prorenin assay, data analysis, draft preparation; Lumbers: supervision, data analysis, draft writing; Pringle: supervision, draft preparation and editing; Roberts: supervision, editing; Zhou: ACE assay; Dekker: clinical management and editing.

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