PORTLAND
PRESS

Research Article

Insulin-regulated aminopeptidase deficiency impairs cardiovascular adaptations and placental development during pregnancy

Sarah L. Walton[1,2], Katrina M. Mirabito Colafella[1,2], Aneesa Ansari[1,2], Siew Yeen Chai[1,3] and
Kate M. Denton[1,2]

[1]Department of Physiology, Monash University, Melbourne, Australia; [2]Cardiovascular Disease Program, Monash Biomedicine Discovery Institute, Monash University, Melbourne, Australia; [3]Neuroscience Program, Monash Biomedicine Discovery Institute, Monash University, Melbourne, Australia

Correspondence: Kate M. Denton (kate.denton@monash.edu)

OPEN ACCESS

Insulin-regulated aminopeptidase (IRAP), an enzyme that cleaves vasoactive peptides including oxytocin and vasopressin, is suggested to play a role in pregnancy and the onset of preeclampsia. Our aim was to examine the contribution of IRAP to arterial pressure regulation and placental development during pregnancy in mice. Mean arterial pressure and heart rate were measured via radiotelemetry in 12-week-old female wild-type and IRAP knockout mice. Females were time-mated with males of the same genotype. Placentae were collected at embryonic day 18.5 for histological analysis. Basal heart rate was ∼40 bpm lower in IRAP knockout females compared with wild-type females. The increase in heart rate across gestation was greater in IRAP knockout females than wild-type females. Neither basal nor gestational mean arterial pressure was different between wildtype and IRAP knockout females. Urine output and water intake of IRAP knockout mice were ∼45% less than wild-type mice at late gestation. IRAP deficiency had no effect on fetal weight. Morphological assessment of placentae revealed that IRAP deficiency was associated with reduced labyrinth surface area and accumulation of glycogen in the junctional zone. Our data demonstrate that IRAP deficiency alters maternal fluid handling and impairs placental labyrinth expansion at late gestation, indicating that IRAP contributes to the normal adaptions to pregnancy.

Introduction

Pregnancy initiates a cascade of profound cardiovascular and renal adaptations, ultimately leading to a rise in cardiac output to ensure adequate oxygen and nutrient supply to the developing fetus [1,2]. Many of these adaptations are mediated by the renin–angiotensin–aldosterone system (RAAS). Normotensive pregnancies are associated with elevated expression of many components of the RAAS, both local and circulating, but also decreased sensitivity to the vasoconstrictive effects of angiotensin (Ang) II [2,3]. Furthermore, the local placental RAAS is active from very early stages of embryogenesis and contributes to physiological processes of the placenta including the regulation of uteroplacental blood flow, angiogenesis and trophoblast invasion [2,3]. Therefore, disturbance of the RAAS equilibrium can interfere with maternal adaptations to pregnancy and placentation, factors that are associated with the pathogenesis of pregnancy disorders such as preeclampsia.

Ang IV is a biologically active fragment of Ang II that binds and competitively inhibits activity of the enzyme, insulin-regulated aminopeptidase (IRAP) [4]. In human placental tissue, IRAP is primarily localized to the syncytiotrophoblasts [5–7] that line the intervillous spaces of the placenta and directly contact maternal blood. Expression of IRAP in syncytiotrophoblasts increases as gestation proceeds [6–8]. In

women, a soluble form of IRAP is present in maternal sera, produced by cleavage from the apical membrane of the syncytiotrophoblasts [9,10]. Serum IRAP activity increases progressively across pregnancy, then plateaus just before birth [11]; this occurs in line with the rapid expansion of the syncytiotrophoblast surface area as the placenta develops. Oxytocin and vasopressin are substrates for IRAP and, for these reasons, IRAP is also known as oxytocinase and vasopressinase [12–14]. This indicates that IRAP plays a role in suppressing levels of these vasoactive peptides in the maternal serum [12,15]. IRAP is also expressed in extravillous trophoblasts (EVTs), which is apparent from late in the first trimester of pregnancy, with expression peaking in the second trimester [8]. EVTs are an invasive cell type that migrate into the maternal decidua and remodel the spiral arteries; a crucial process for establishing the maternal–fetal circulation. IRAP has been shown to promote invasion and proliferation of trophoblasts in *in vitro* settings [8,16]. These studies provide evidence of a role for IRAP in the maternal cardiovascular adaptations to pregnancy and also placental development.

Aberrant IRAP regulation has been implicated in pregnancy complications such as preterm birth and preeclampsia. Reduced serum IRAP activity has been reported in women who experienced spontaneous preterm delivery [17], and also in preeclamptic women compared with normotensive women [12,18]. Furthermore, IRAP expression at both the mRNA and protein level is significantly lower in placental tissue from women with preeclampsia, compared with normal term tissue [7]. Although the underlying mechanisms remain to be elucidated, low placental IRAP expression may contribute to the failure of EVTs to invade deep into the maternal decidua, resulting in inadequate spiral artery remodelling observed in preeclampsia [19]. Inadequate expression of IRAP in the placenta could allow leakage of vasoactive peptides into the maternal circulation, thus increasing the risk of preterm delivery and preeclampsia.

To date, little is known about the role of IRAP in mediating cardiovascular adaptions and placental development during pregnancy. To address this, we examined pregnancy outcomes in IRAP-deficient female mice. We hypothesized that IRAP has a beneficial contribution to the regulation of arterial pressure during pregnancy and placentation. Our aims were to determine (1) the contribution of IRAP to the regulation of arterial pressure during pregnancy and (2) the impact of IRAP deficiency on placental development and fetal outcomes at late gestation.

Methods

Animals

All experiments were approved by the Monash Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Animal experiments were conducted at Monash University (Clayton, VIC, Australia). Wild-type (WT) and IRAP knockout (KO) mice were generated as described previously [20] and adult mice of 10–12 weeks of age were used. All mice were housed in an experimental room with temperature maintained at 25°C and a 12 h light–dark cycle. Nulliparous females were randomly allocated to either blood pressure monitoring by radiotelemetry (WT: $n=5$, IRAP KO: $n=5$) or analysis of renal excretory function, and placental and fetal growth (WT: $n=8$, IRAP KO: $n=8$).

Blood pressure

Female mice were anaesthetized (isoflurane; 2–5% v/v O_2 in N_2), and a radiotelemetry probe (PA-C10, Data Sciences International, MN, U.S.A.) was implanted into the left carotid artery for the measurement of arterial pressure, heart rate and activity as previously described [21]. After a 10-day recovery period, baseline arterial pressure, heart rate (HR) and locomotor activity were recorded for 3 days. Female mice were then time-mated with a non-telemetered male of the same genotype. The presence of a vaginal plug was deemed embryonic day (E) 0.5, and the breeding pair was then separated. Arterial pressures, HR and locomotor activity were measured, as described above, throughout gestation.

Renal excretory function

A separate cohort of nulliparous female mice (non-telemetered) was used to study urinary excretion, and fetal and placental morphology. Before mating (baseline) and at E17.5 of pregnancy, female mice were placed in individual glass metabolic cages to collect 24 h urine samples. All mice were acclimatized to the cages prior to beginning urine collection. Food and water consumption, and body weight change were recorded over 24 h. Urine samples were collected and stored at −20°C prior to analysis of total protein content via the Bradford assay and osmolality via freezing point depression.

Tissue collection

Immediately following removal from the metabolic cage on E18.5, mice were deeply anaesthetized with isoflurane and exsanguinated via cardiac puncture. Plasma osmolality was determined via freezing point depression. The numbers of viable and resorbing implantation sites were counted. Fetuses and placentae were removed, weighed and dimensions recorded. From each dam, placentae from one male and one female fetus were hemisected and fixed in 4% PFA for histological analysis. The maternal kidneys and hearts were weighed and fixed in 4% PFA.

Cardiomyocyte histology

Hearts were processed to paraffin and cross-sectional sections were collected at 4 μm. Sections were stained with H&E, scanned using the Aperio slide scanner (Leica Microsystems Pty Ltd, VIC, Australia), and cardiomyocyte areas (60/animal) were measured using Aperio ImageScope software.

Placental morphometry

Placentae were processed to paraffin and 4 μm thick mid-sagittal sections were collected for H&E staining or immunohistochemistry (Monash Histology Platform, VIC, Australia). Slides stained with H&E were scanned using the Aperio slide scanner. Labyrinth, junctional zone and glycogen cell cross-sectional areas were determined using Aperio ImageScope software [22]. Fetal capillaries and trophoblast cells in the placental labyrinth were detected by dual immunohistochemistry with anti-vimentin and anti-cytokeratin, respectively, as previously described [23]. Areas of trophoblast, fetal blood space and maternal blood space were determined via point counting five randomly-selected images (320 points per image) of the placental labyrinth zone.

Immunohistochemical detection of IRAP and AQP2

The placental slides and maternal kidney slides were dewaxed as described above and subjected to heat-mediated antigen retrieval in citrate buffer (pH 6.0) at 95°C for 20 min. Slides were treated with 0.3% hydrogen peroxide to quench endogenous peroxidases and blocked in 10% goat serum diluted in blocking buffer (#S0809, Agilent Dako, CA, U.S.A.) to prevent non-specific binding. Placental slides were incubated with an anti-rabbit IRAP antibody (1:500 dilution, #6918, Cell Signaling Technology, MA, U.S.A.) and maternal kidney slides were incubated with an anti-rabbit AQP2 antibody (1:1000 dilution; AB3274, Sigma Aldrich, NSW, Australia) for 1 h at room temperature Slides were washed in PBS and then incubated with a biotinylated rabbit secondary antibody for 45 min at room temperature (1:200 dilution, #BA-1000, Vector Laboratories, CA, U.S.A.), washed in PBS and incubated in avidin–biotin enzyme complex (Elite ABC Kit, #PK-6100, Vector Laboratories) for 30 min. Colour was developed with DAB and counter stained with haematoxylin. Renal AQP2 expression was analysed as previously described [24].

Statistics

All data are presented as mean $\pm$ standard error of mean (SEM) unless indicated otherwise. Two-way ANOVA was used to analyse maternal metabolic cage data, fetal data (fetal weights, dimensions, organ weights) and placental data (placental weights, dimensions, morphology) with the factors genotype and time/sex. Maternal baseline 24 h arterial pressures, HR and locomotor activity and biometry were analyzed using Student's *t*-tests. During pregnancy, arterial pressures, HR and locomotor activity were analyzed using repeated measures ANOVA with the factors genotype and time. Sidak *post hoc* tests were performed where appropriate. A P value of $\alpha < 0.0.5$ was considered statistically significant.

Results

Baseline haemodynamics

MAP (WT: 101 ± 2 mmHg, IRAP KO: 101 ± 1 mmHg, $P=0.96$, Figure 1A), systolic blood pressure (SBP, Figure 1C) and diastolic blood pressure (DBP, Figure 1E) over the 3-day baseline period were similar between WT and IRAP KO females. By contrast, HR was ~40 bpm lower in IRAP KO mice compared with WT mice at baseline (WT: 515 ± 11 bpm, IRAP KO: 475 ± 12 bpm, $P=0.04$, Figure 1G). Baseline locomotor activity was not statistically different between genotypes ($P=0.21$, Figure 1I).

Haemodynamics during pregnancy

Arterial pressures, HR and activity levels across pregnancy are presented in absolute values in Supplementary Figure S1, and relative to baseline recordings in Figure 1. Pregnancy was associated with a mid-gestational dip in MAP in
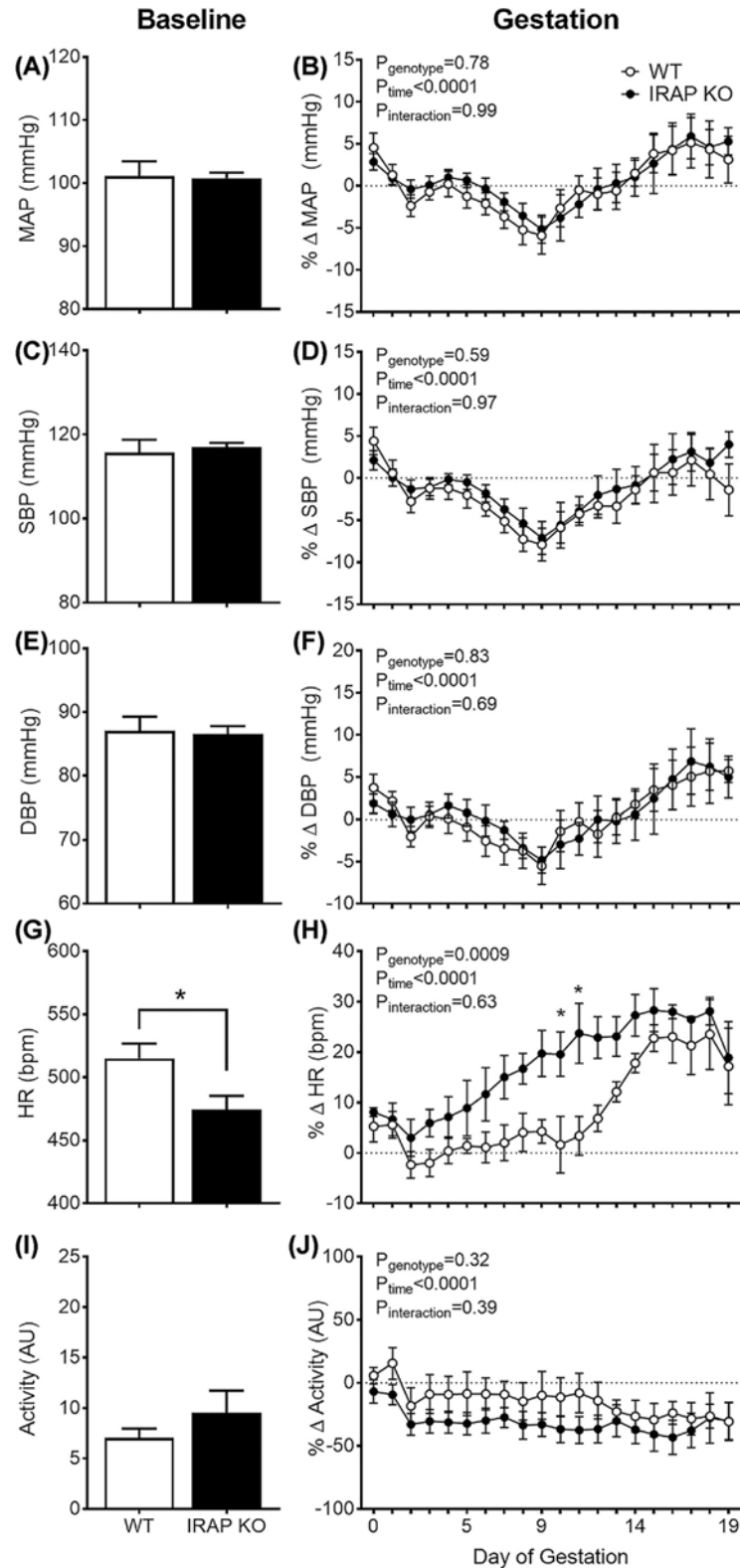
Figure 1. Effect of IRAP deficiency on arterial pressure and heart rate during pregnancy

(**A,B**) Mean arterial pressure (MAP), (**C,D**) systolic blood pressure (SBP), (**E,F**) diastolic blood pressure (DBP), (**G,H**) heart rate (HR) and (**I,J**) locomotor activity at baseline (left) and the change (%) from mean baseline levels (right) in WT (open bars; $n=5$) and IRAP KO (closed bars; $n=5$) mice. Data presented as mean $\pm$ SEM. Baseline data analyzed via Student's t-test. Relative change data analyzed via repeated measures ANOVA with a Sidak's multiple comparisons test. *$P<0.05$ vs WT.
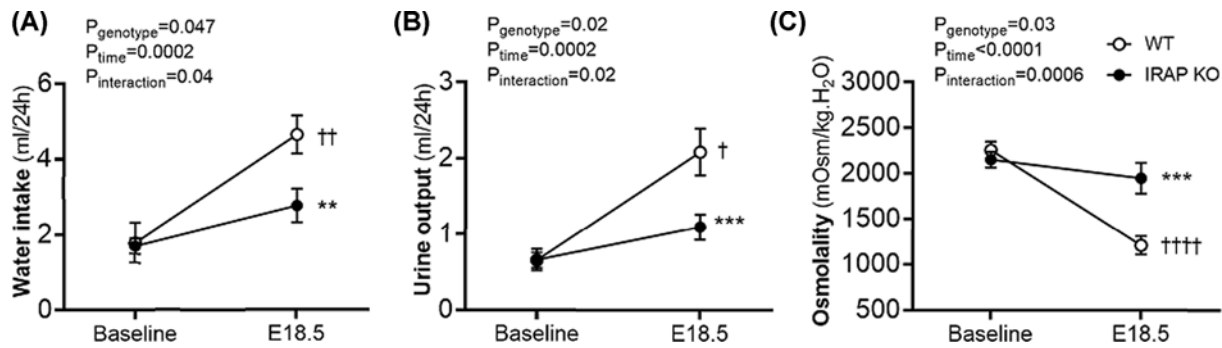
PORTLAND
PRESS

Figure 2. Effect of IRAP deficiency on fluid balance during pregnancy
(**A**) Water intake, (**B**) urine output and (**C**) urine osmolality over 24 h at baseline and late pregnancy (E18.5) in WT (open points; $n=8$) and IRAP KO (closed points; $n=8$) mice. Data presented as mean $\pm$ SEM. Data analyzed via repeated measures ANOVA with a Sidak's multiple comparisons test. **$P<0.01$, ***$P<0.001$ comparing E18.5 IRAP KO with E18.5 WT. †$P<0.05$, ††$P<0.01$, ††††$P<0.0001$ comparing E18.5 WT with baseline WT.

both WT and IRAP KO mice, with the nadir occurring at day 9 of pregnancy ($P_{time}<0.0001$, Figure 1B). MAP then increased above pre-pregnancy levels by ~7% in both genotypes during late gestation. No differences in SBP and DBP were observed between genotypes during pregnancy (Figure 1D,F). HR increased significantly across pregnancy in both WT and IRAP KO mice ($P_{time}<0.0001$, Figure 1H). However, the increase in HR was greater in IRAP KO mice than WT mice ($P_{genotype} = 0.003$, Figure 1H) in mid-gestation. Locomotor activity declined as pregnancy progressed and this occurred to a similar extent between the genotypes (Figure 1J).

Fluid balance and proteinuria in pregnancy
There were no significant differences in water intake, urine output or urine osmolality between non-pregnant WT and IRAP KO females (Figure 2). Pregnant WT females drank more water (+160%, $P<0.01$, Figure 2A) and produced greater volumes of urine (+218%, $P<0.05$, Figure 2B) of lower osmolality (−46%, $P<0.0001$, Figure 2C) compared with non-pregnant WT values. By contrast, IRAP KO mice did not exhibit the pregnancy-associated increases in water intake (Figure 2A) and urine output (Figure 2B) that were observed in WT mice. IRAP KO mice produced urine of greater osmolality (+60%, $P<0.001$, Figure 2C) than WT mice at E18.5. These data indicate that IRAP KO mice produced smaller volumes of more concentrated urine compared with WT mice at E18.5. Plasma osmolality was not different between WT and IRAP KO mice at E18.5 (WT: 307 ± 7 mOsm/kg.H_2O; IRAP KO: 312 ± 9 mOsm/kg.H_2O; $P=0.7$). Baseline urinary protein excretion was similar between genotypes. At E18.5, IRAP KO produced urine with greater total protein concentration than WT mice ($P<0.001$, Figure 3A). However, total protein excretion over 24 h was not significantly different between the genotypes (Figure 3B).

AQP2 expression in maternal kidneys
The proportion of inner and outer medulla positively stained for AQP2 was greater in kidneys from IRAP KO females compared with WT at E18.5 (Figure 4A,C). Intense AQP2 expression was concentrated on the apical surface and throughout the cytoplasm of the collecting duct epithelial cells in IRAP KO mice. The proportion of renal tissue positive for AQP2 in the renal cortex was similar between genotypes. Pearson's correlation coefficient was used to determine whether the enhanced urine concentrating capacity of pregnant IRAP KO mice was associated with increased AQP2 expression in the inner medulla, the final site of water reabsorption in the kidney. Overall, there was a positive correlation between the proportion of AQP2 staining in the inner medulla and urine osmolality at E18.5 (Figure 4B).

Maternal, fetal and placental outcomes
Maternal body weight gain during pregnancy was similar between WT and IRAP KO mice (Table 1). Total maternal kidney weight was similar between the genotypes. Heart weight, both absolute and corrected for body weight, of IRAP KO dams was ~14% less than WT dams at E18.5 (Figure 5A,B; $P<0.05$). In a separate cohort of young, non-pregnant female mice, heart (Figure 5A,B) and kidney weights (WT, 239 ± 11 mg vs. IRAP KO, 281 ± 19 mg, $P=0.4$) were similar between the genotypes. Further histological analysis of maternal hearts collected at E18.5 revealed cardiomyocyte surface area was significantly less in IRAP KO mice compared to WT mice (Figure 5C–E).
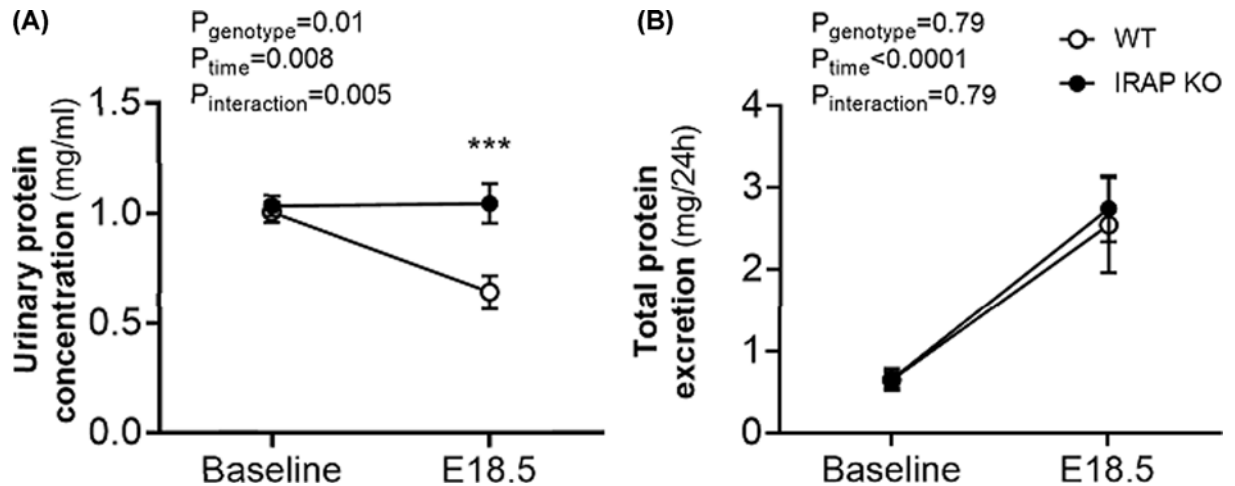
Figure 3. Effect of IRAP deficiency on proteinuria during pregnancy
(**A**) Urinary protein concentration and (**B**) total protein excretion over 24 h at baseline and late pregnancy (E18.5) in WT (open points; $n=8$) and IRAP KO (closed points; $n=8$) mice. Data presented as mean $\pm$ SEM. Data analyzed via repeated measures ANOVA with a Sidak's multiple comparisons test. ***$P<0.001$ comparing E18.5 IRAP KO with E18.5 WT.

Table 1 Maternal biometry at E18.5

	WT	IRAP KO
E0 BW (g)	20.5 ± 0.5	21.3 ± 0.8
E18.5 BW (g)	37.4 ± 0.8	38.4 ± 1.5
Total kidney (mg)	357.8 ± 21.1	333.7 ± 14.5
Total kidney:BW	9.7 ± 0.6	9.0 ± 0.2
Total conceptus weight (g)	8.32 ± 0.87	8.22 ± 0.84
Litter size	7.3 ± 0.7	7.3 ± 0.8
Male fetuses (%)	50.9 ± 7.5	46.9 ± 7.5
Resorptions (number)	0.2 ± 0.2	1.1 ± 0.4

Data expressed as mean $\pm$ SEM. $N=8$ dams/genotype. Data analyzed by Student's t-tests. Total conceptus weight is the sum of all fetuses and placentas in each litter. BW, body weight. E, embryonic day.

Litter size, total conceptus weight, the number of fetal resorptions per pregnancy and fetal sex ratios were similar between genotypes (Table 1). Both length and width of placentas collected from IRAP KO were marginally less than that of WT mice ($P_{genotype}<0.05$, Table 2). However, placental weight and placental efficiency (the ratio of placental to fetal weight) were not different between the genotypes. There were no significant differences between the genotypes with regard to fetal body and organ weights (Table 2).

Expression of IRAP in placentae
Immunohistochemical labelling showed that IRAP protein was localized to the labyrinthine syncytiotrophoblasts in WT mice (Figure 6, arrows). Staining was most intense in the apical membrane, although cytoplasmic IRAP staining can be seen in most syncytiotrophoblasts. IRAP was not detected in the endothelial cells lining the fetal blood spaces (Figure 6, asterisks). Within the junctional zone, there was diffuse cytoplasmic staining for IRAP in the parietal trophoblast cells (Figure 6, arrowheads) while the spongiotrophoblasts demonstrated no immunostaining. Intense IRAP expression was observed in the endothelial cells lining the maternal blood sinuses in the junctional zone (Figure 6, hash). As expected, staining for IRAP was completely absent in placentae from IRAP KO mice (Figure 6, inset).

Placental morphology
H&E staining showed no gross morphological differences between placentae collected from WT and IRAP KO mice (Figure 7J,K). In the mouse placenta, the labyrinth is the site of nutrient and waste exchange between the fetal and maternal blood supplies, and the junctional zone functions as an endocrine layer. Shifts in the proportion of labyrinth
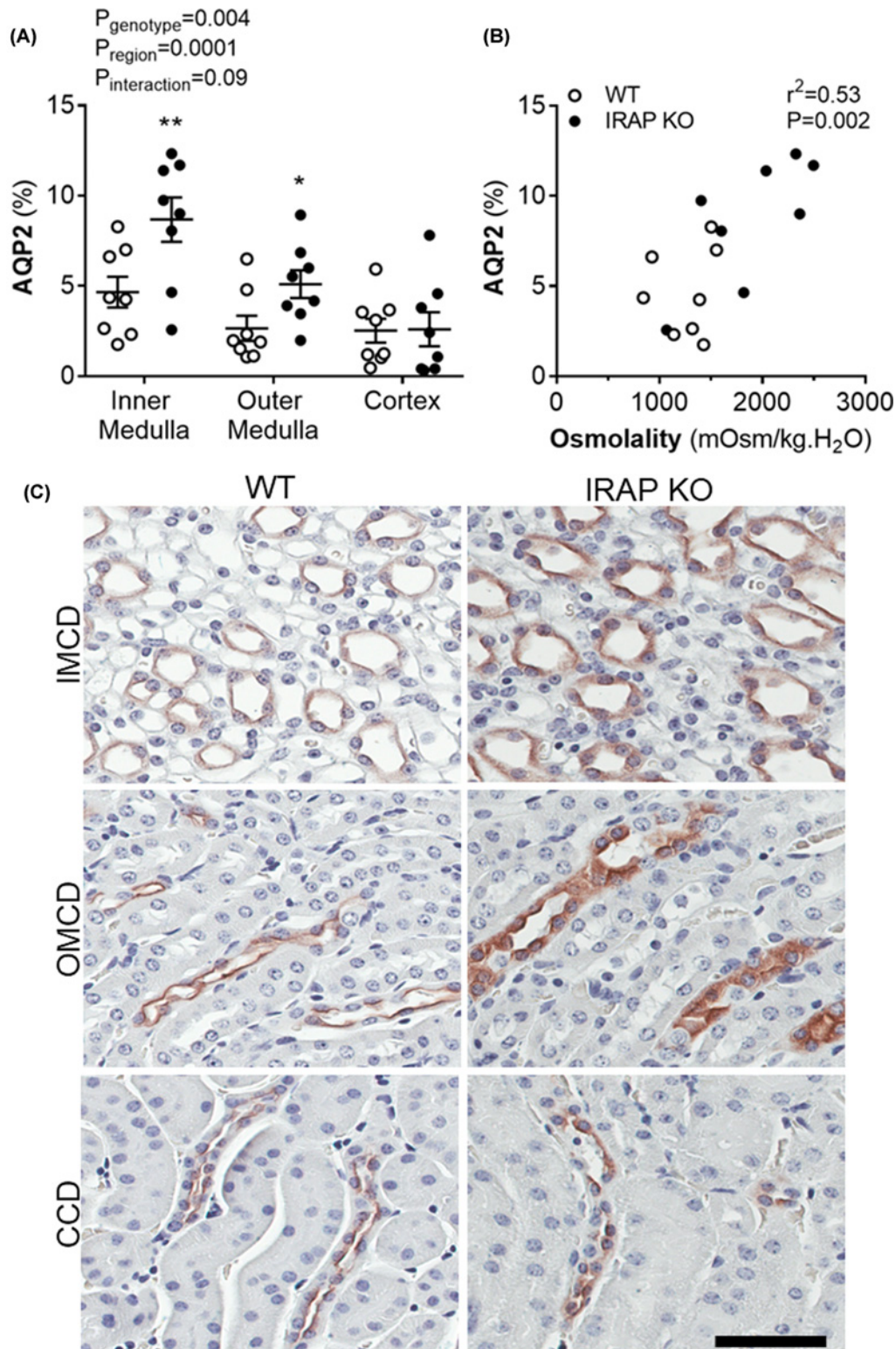
Figure 4. Immunohistochemical detection of AQP2 in maternal kidneys

(**A,C**) AQP2 staining (% area) in the inner medulla, outer medulla and cortex of kidneys collected from WT (open points; $n=8$) and IRAP KO (closed points; $n=8$) mice at E18.5. (**B**) Correlation analysis between AQP2 expression in the inner medulla and urine osmolality at E18.5. Data presented as mean $\pm$ SEM. (A) Data analyzed via two-way ANOVA with a Sidak's multiple comparisons test. *$P<0.05$, **$P<0.01$ comparing WT and IRAP KO. (B) Data analyzed using Pearson's correlation. IMCD, inner medullary collecting duct; OMCD, outer medullary collecting duct; CCD, cortical collecting duct.
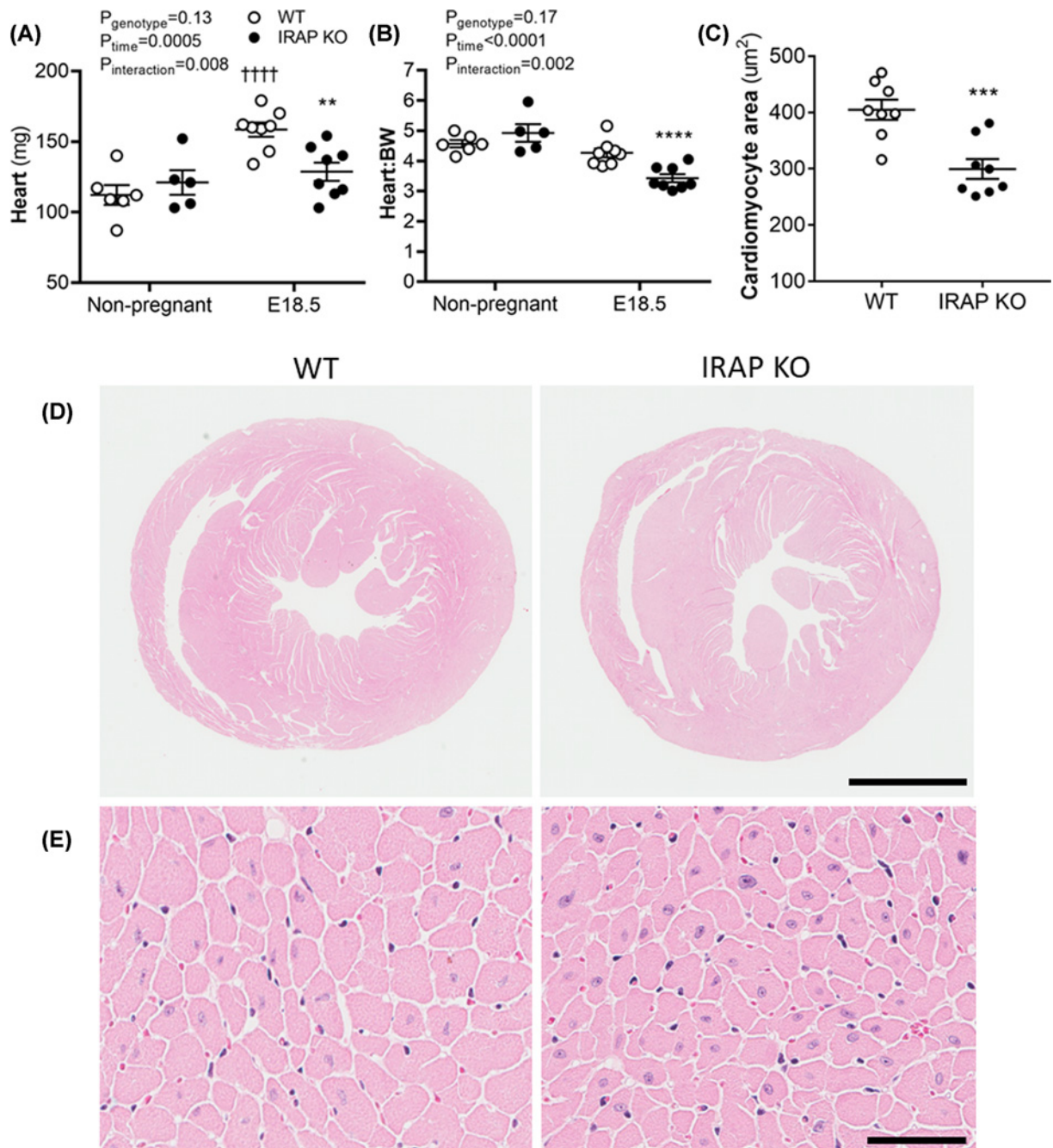
Figure 5. Effect of IRAP deficiency on pregnancy-induced cardiac hypertrophy

(**A**) Heart weight and (**B**) heart weight relative to body weight in non-pregnant and pregnant (E18.5) WT (open points) and IRAP KO mice (closed points). (**C**) Cardiomyocyte area in WT and IRAP KO mice at E18.5. H&E staining of hearts at E18.5 in low (**D**, scale = 2mm) and high (**E**, scale = 50 μm) power. Data expressed as mean $\pm$ SEM. Non-pregnant: N=5-6/genotype. E18.5: N=8 dams/genotype. (A and B) Data analyzed via two-way ANOVA with a Sidak's multiple comparisons test. $**P<0.01$, $***P<0.001$, $****P<0.0001$ comparing E18.5 IRAP KO with E18.5 WT. $^{\dagger\dagger\dagger\dagger}P<0.0001$ comparing E18.5 WT with baseline WT. (C) Data analyzed by Student's *t*-test with $***P<0.001$. BW, body weight. E, embryonic day.

Table 2 Fetal and placental biometry at E18.5

	WT		IRAP KO		Statistics
	Male	**Female**	**Male**	**Female**	
Fetal weight (g)	1.05 ± 0.03	0.98 ± 0.03	1.01 ± 0.02	0.99 ± 0.04	$P_{\text{genotype}} = 0.71$ $P_{\text{sex}} = 0.16$
Snout-rump (mm)	24.76 ± 0.22	23.96 ± 0.36	24.59 ± 0.34	24.10 ± 0.47	$P_{\text{genotype}} = 0.97$ $P_{\text{sex}} = 0.09$
Head length (mm)	9.78 ± 0.05	9.52 ± 0.11	9.63 ± 0.08	9.39 ± 0.14	$P_{\text{genotype}} = 0.20$ $\boldsymbol{P_{\text{sex}} = 0.03}$
Liver weight (mg)	63.1 ± 4.0	58.9 ± 2.4	60.8 ± 3.0	59.1 ± 2.7	$P_{\text{genotype}} = 0.73$ $P_{\text{sex}} = 0.34$
Brain weight (mg)	67.6 ± 1.7	63.5 ± 1.6	62.9 ± 1.8	62.2 ± 1.8	$P_{\text{genotype}} = 0.10$ $P_{\text{sex}} = 0.18$
Placental weight (mg)	138.3 ± 6.6	128.0 ± 8.0	131.6 ± 3.1	129.7 ± 5.5	$P_{\text{genotype}} = 0.53$ $P_{\text{sex}} = 0.23$
Placental efficiency (g BW:mg placenta)	7.74 ± 0.05	8.16 ± 0.59	7.84 ± 0.20	7.79 ± 0.42	$P_{\text{genotype}} = 0.77$ $P_{\text{sex}} = 0.68$
Placental length (mm)	8.93 ± 0.24	8.77 ± 0.16	8.71 ± 0.13	8.28 ± 0.14	$\boldsymbol{P_{\text{genotype}} = 0.04}$ $P_{\text{sex}} = 0.09$
Placental width (mm)	8.40 ± 0.19	8.34 ± 0.14	8.19 ± 0.10	7.83 ± 0.15	$\boldsymbol{P_{\text{genotype}} = 0.02}$ $P_{\text{sex}} = 0.19$
Placental depth (mm)	1.96 ± 0.06	1.93 ± 0.08	2.03 ± 0.08	2.04 ± 0.05	$P_{\text{genotype}} = 0.20$ $P_{\text{sex}} = 0.93$

Data expressed as mean $\pm$ SEM. $N=8$ litters/genotype. Data analyzed by two-way ANOVA with the factors genotype and sex; no significant interactions were found. BW, body weight.

and junctional zones can indicate alterations in placental efficiency. Cross-sectional analysis showed total placental area was ∼8–9% less in IRAP KO compared with WT mice ($P_{\text{genotype}} = 0.01$, Figure 7A). There was an overall effect of genotype for labyrinth area ($P_{\text{genotype}} = 0.003$, Figure 7B), and post-hoc analysis revealed that this effect was greatest in male offspring ($P<0.01$, Figure 7B). Labyrinth area relative to total placental area was lower in male IRAP KO compared with male WT ($P<0.05$, Figure 7C). This may suggest a subtle decrease in placental efficiency in IRAP KO mice, particularly in males. Junctional zone area was unaffected by IRAP deficiency (Figure 7D), indicating a disproportionate shift in placental architecture. The cross-sectional area of glycogen cells, both as an absolute area and expressed as a percentage of total placental area, was ∼1.6- to 1.8-fold greater in IRAP KO placentae compared with WT placentae (Figure 7E,F; $P_{\text{genotype}}<0.01$). Further quantification of labyrinth structure showed no significant differences for the proportion of trophoblasts, fetal blood space or maternal blood space occupying the placental labyrinth between the genotypes (Figure 7G–I).

Discussion

The RAAS is a highly complex and integrative system, and disruption to specific components can contribute to disorders of pregnancy including preeclampsia. The activity of IRAP can be regulated by components of the RAAS as Ang IV has been found to be a competitive inhibitor of the enzyme. Moreover, IRAP is thought to play a role in regulating the circulating levels of the peptide hormone, oxytocin, during pregnancy. To further characterize the role of IRAP in pregnancy, we used IRAP KO mice to examine maternal haemodynamic adaptations and fetal/placental outcomes. Our experiments demonstrate two important findings: (1) IRAP has a clear role in the maternal cardiovascular adaptions to pregnancy and (2) IRAP regulates placental labyrinth development in mice. Understanding how IRAP influences cardiovascular function and placental development may be used to improve pregnancy outcomes.

IRAP deficiency alters maternal heart rate response to pregnancy

Non-pregnant IRAP KO females had lower resting heart rate compared with WT counterparts. Cardiac output is a function of stroke volume and heart rate, suggesting either cardiac output was lower in non-pregnant IRAP-KO females or cardiac output was maintained in IRAP KO females via increases in stroke volume. Currently we cannot delineate between these two possibilities and future echocardiography would be warranted to assess cardiac function. However, an increase in stroke volume and normalization of cardiac output might suggest an increase in venous return or contractility. The alternative is that IRAP deficiency is associated with increased total peripheral resistance. In normal pregnancy, the progressive increase in blood volume triggers marked physiological adaptions including
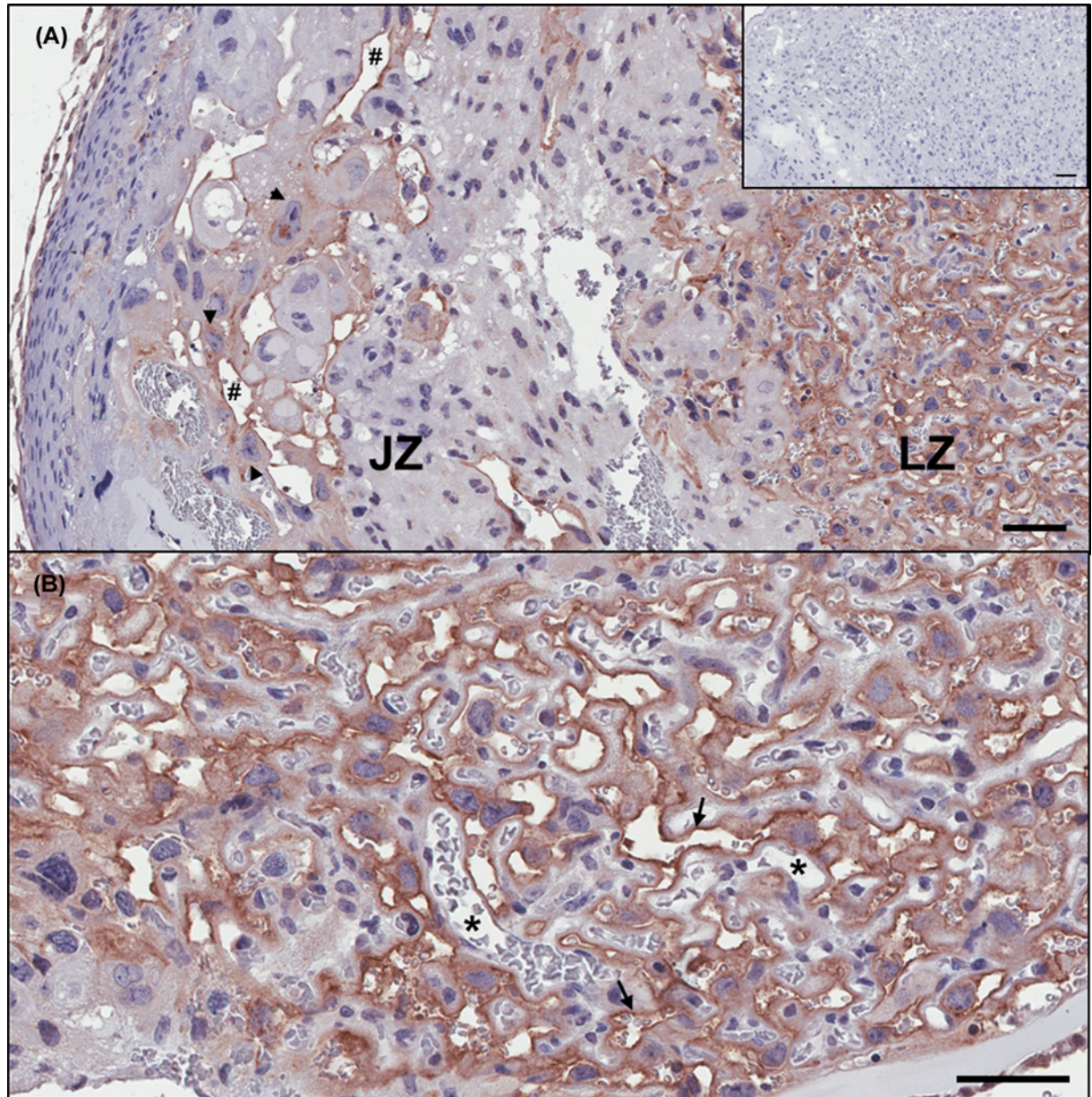
Figure 6. Immunohistochemical detection of IRAP in placentas from WT mice at E18.5
(**A**) IRAP staining in the junctional zone (JZ) and labyrinth zone (LZ) of the WT placenta. Arrowheads point to maternal blood sinuses in the JZ. Inset shows no IRAP staining is evident in the IRAP KO placenta. (**B**) Higher magnification of the LZ. Asterisks show fetal blood spaces and arrows point to syncytiotrophoblasts lining the maternal blood spaces; scale, 50 μm.

increased cardiac output, and decreases in arterial pressure and systemic vascular resistance [25–28]. The increase in cardiac output in women [27] and mice [29] is achieved primarily through increases in stroke volume, and to a lesser extent, increases in HR. The persistent volume overload in pregnancy results in marked ventricular hypertrophy. Failure to make or sustain these adaptations can contribute to hypertensive disorders of pregnancy [26]. In our study, IRAP-deficient dams displayed a greater mid-gestational increase in HR compared with WT counterparts, although arterial pressure was similar between the genotypes. Hypertensive pregnancies are associated with enhanced sympathetic regulation of HR [30,31] and impaired blood volume expansion [26,32,33] compared with normotensive pregnancies. A greater HR response in IRAP-deficient dams could be interpreted as a compensatory response to a failure to increase blood volume in pregnancy similar to what is observed in hypertensive pregnancy disorders. [32,33] Indeed, pregnancy-associated cardiac hypertrophy was absent in IRAP-deficient dams, as shown via
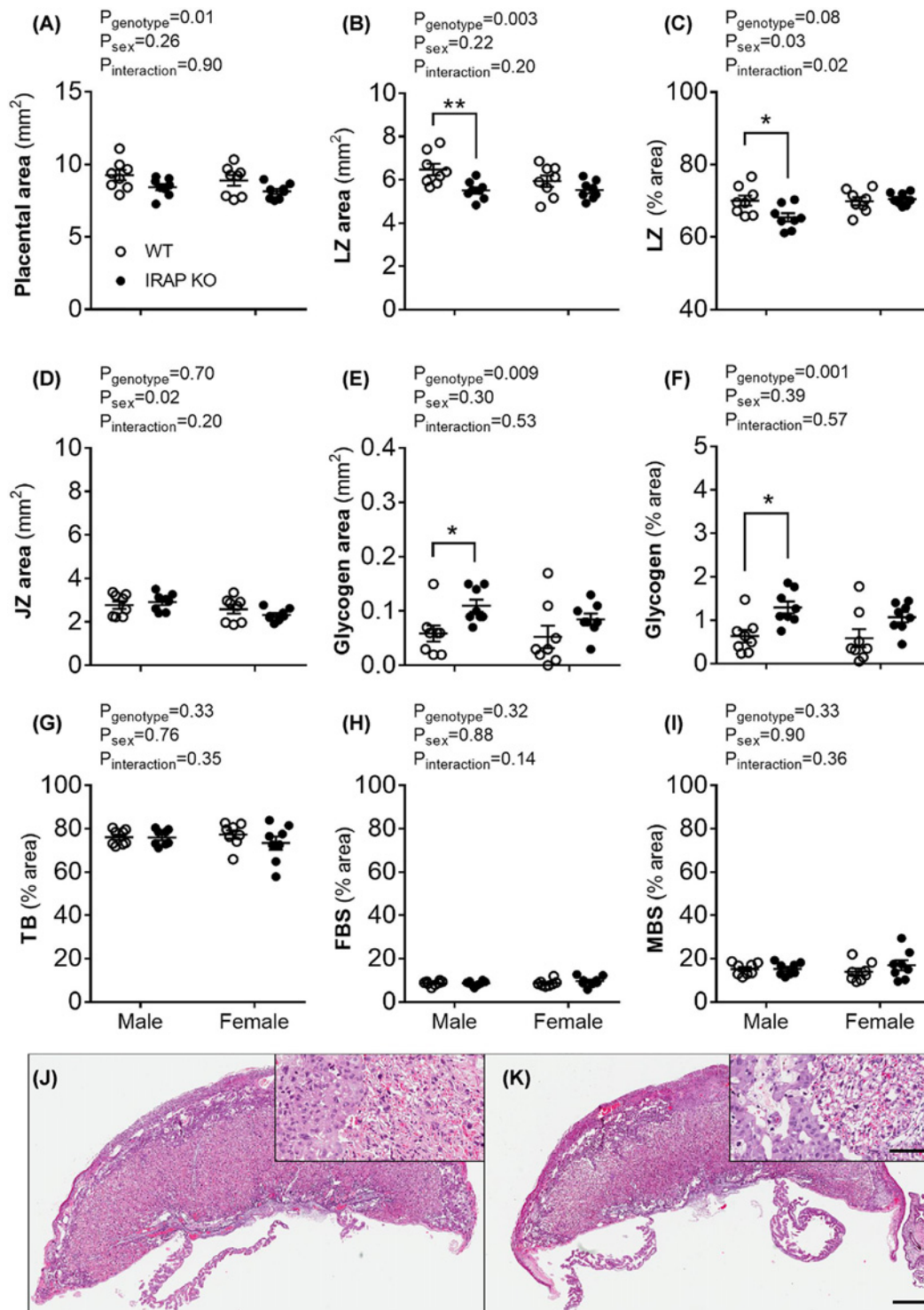
Figure 7. Comparison of placental architecture between WT and IRAP KO mice

(**A**) Total placenta cross-sectional area. (**B**) Labyrinth zone (LZ) cross-sectional area. (**C**) LZ area as a percentage of total placenta area. (**D**) Junctional zone (JZ) cross-sectional area. (**E**) Glycogen cell cross-sectional area. (**F**) Glycogen area as a percentage of total placenta area. The proportion of (**G**) trophoblast, (**H**) fetal blood space (FBS) and (**I**) maternal blood space (MBS) occupying the placental labyrinth zone. Representative H&E sections of WT (**J**) and IRAP KO (**K**) placentas from male offspring (scale, 500 μm). Inset shows higher magnification of LZ and JZ with glycogen stores appearing white; scale, 50 μm). WT (open points; $n=8$) and IRAP KO (closed points; $n=8$) mice. Data presented as mean $\pm$ SEM. Data analyzed via two-way ANOVA with a Sidak's multiple comparisons test. *$P<0.05$, **$P<0.01$ comparing WT and IRAP KO males.

smaller cardiomyocyte surface area, suggesting that IRAP facilitates the increase in blood volume observed in normal pregnancy. Echocardiography and sympathetic nerve activity studies could provide comprehensive information on pregnancy-associated cardiovascular changes in IRAP-deficient pregnancies in the future.

IRAP deficiency alters water balance during pregnancy

Pregnancy produces marked changes in renal physiology, including significant vasodilation, and increased glomerular filtration rate and renal blood flow [11,34]. The osmotic threshold for vasopressin stimulation is lowered, leading to the mild hyponatremia hypervolemia observed in pregnancy [2,11]. In our study, pregnancy-induced increases in water intake and urine output in IRAP deficient dams were almost entirely suppressed. This shows a clear role for IRAP in the regulation of fluid balance in pregnancy. IRAP is highly expressed in the distal tubules and principal cells of the collecting ducts [35,36], the site of final water reabsorption in the kidney. In the principal cells, vasopressin (a substrate of IRAP) stimulates the translocation of aquaporin (AQP)-2 from subcellular vesicles to the apical membrane to reabsorb water. The marked upregulation of AQP2 expression in the renal medulla of pregnant IRAP KO mice is indicative of V_2-receptor mediated vasopressin action in the kidney to increase water reabsorption. Our findings agree with previous studies showing plasma vasopressin [13] and renal AQP-2 protein levels [36] are two-fold greater in non-pregnant IRAP KO mice. Although these mice are able to maintain basal fluid homeostasis, it appears the additional challenge of pregnancy unmasks a fluid-retaining phenotype. In line with our findings, vasopressin administration in late pregnancy increased urine concentrating capacity to a greater extent in IRAP KO mice compared to WT mice [37]. This suggests pregnant IRAP KO mice have greater sensitivity to the effects of vasopressin and/or higher circulating vasopressin levels than pregnant WT mice.

Evidence for an optimal level of maternal hydration remains scant, despite increased water intake being critical for normal expansion of plasma volume, amniotic fluid production and fluid exchange between mother and fetus [11,38,39]. A 24-h urine concentration less than 500 mOsm.kg^{-1} has been suggested as ideal in pregnant women [40]. This highlights adequate water intake is important for both the mother and the fetus. Furthermore, elevated maternal plasma vasopressin (a potent vasoconstrictor) is considered a biomarker of preeclampsia in women [41]. Key features of preeclampsia, including pregnancy-specific hypertension, proteinuria, renal injury and fetal growth restriction, can be recapitulated in the mouse via chronic infusion of vasopressin [41]. Given our findings suggest IRAP deficient dams have elevated vasopressin levels in pregnancy, it is surprising that we did not observed these common features of preeclampsia. A secondary insult such as chronic hypertension or diabetes may unmask a phenotype resembling an adverse pregnancy outcome such as preeclampsia in these animals.

IRAP is highly expressed in placental syncytiotrophoblasts at late gestation

As shown in our study and in human tissue, IRAP protein is intensely expressed in the apical membrane of syncytiotrophoblasts in the placenta at late gestation [5,7,8]. In the mouse, the syncytiotrophoblasts act as an exchange barrier between the fetal capillaries and maternal blood sinusoids. The mechanism of IRAP action in the placenta is unclear. However, IRAP's localization to the vasculature and ability to degrade vasoactive peptides hints towards roles in placental blood flow regulation or degradation of waste products. It is important to note that mice lack the cleavage site for the release of soluble IRAP as seen in humans, and therefore soluble IRAP is not present in sera from pregnant mice [42]. This indicates membrane-bound IRAP exerts effects locally at the tissue level in mice, rather than in the serum. Interestingly, IRAP protein was not detected in the fetal capillaries. Labyrinth efficiency is determined in-part by the organisation of its highly complex vasculature network. Differentiating between maternal blood spaces and fetal capillaries can identify where impairments arise, although finding adequate labelling techniques is challenging [43]. IRAP is an excellent candidate for immunolabelling the labyrinth vasculature, being significantly faster and more cost-effective than traditional techniques using ultrathin resin sections or double-labelled paraffin sections [23,43].

IRAP deficiency perturbs placental development at late gestation

Impaired placental labyrinth formation, as observed in placentas from IRAP-deficient males, is an indicator of poor placental function [44]. There is substantial evidence to suggest that IRAP can directly regulate placental development. Firstly, IRAP protein is highly expressed EVTs [7,8] that invade the maternal decidua and surround and remodel spiral arteries in early pregnancy. Deficient IRAP expression in the EVT cell population has been reported in placentas from preeclamptic women [7], which may contribute to shallow trophoblast invasion of the spiral arteries. Serum IRAP is also low in women with preeclampsia [18], likely reflecting impaired trophoblast growth. Evidence

from *in vitro* studies indicates IRAP can modulate the invasive properties of trophoblasts. Forskolin-induced differentiation of BeWo choriocarcinoma cells, a model where normal placental trophoblasts are differentiated into syncytiotrophoblasts, was associated with upregulation of IRAP mRNA [45,46]. Cohen and colleagues [16] showed several inhibitors of IRAP (LVV-hemorphin 7 and angiotensin peptides: divalinal AngIV and norleucine-1-Ang IV) reduced cytotrophoblast invasiveness via regulation of metalloproteinase-9 activity. Although high expression of IRAP in the placenta appears critical during placentation, uterine or umbilical artery Doppler flow are required to understand the functional significance of a smaller placental labyrinth.

Fetal weight was not affected by IRAP deficiency despite the reduction in labyrinth area, indicating compensatory mechanisms were sufficient to maintain fetal growth. For example, the complexity of the fetal/maternal vascular organisation within the labyrinth was similar between the genotypes. This may allow for sufficient gas and nutrient exchange between mother and placenta. The close proximity of glycogen trophoblasts to the vasculature suggests glycogen may exist as a source of glucose for the rapid portion of fetal growth in late gestation [47,48]. Therefore, the excess glycogen deposition, considered an indicator of developmental disturbance [47,48], could have aided in maintaining fetal growth in the setting of IRAP deficiency.

Of interest, IRAP deficiency primarily affected placental structure of male but not female offspring. This could reflect sex differences in IRAP expression/activity in the mouse placenta. Evidence exists for sex differences in other RAAS-associated serum peptidases including APA and ACE [49] but, to our knowledge, sex differences in IRAP have not been reported. Furthermore, the placenta possesses sex-specific structural and functional characteristics that influence how a developing fetus responds to a sub-optimal environment [50]. Males are reported as having more severe outcomes in terms of placental development and fetal morbidity/mortality than females in both humans [50] and rodents [51]. This suggests sex-specific adaptations of the placenta to a stress, such as IRAP deficiency, may be a key determinant of offspring outcomes. These are areas of interest for future studies.

In summary, our study provides the first evidence that deficiency of IRAP, an enzyme associated with the RAAS, alters the maternal cardiovascular response to pregnancy and impairs placental development in mice. Aberrant activity of RAAS components, including IRAP, has been observed in human pregnancies complicated by adverse pregnancy outcomes such as preterm birth and preeclampsia. This highlights the need for further work to elucidate the contribution of IRAP to maternal cardiovascular health and adverse pregnancy outcomes observed in humans.

Clinical perspectives

- Aberrant activity of the renin–angiotensin aldosterone system has been implicated in the pathogenesis of pregnancy disorders such as preeclampsia, but the involvement of associated smaller peptide fragments such as insulin-regulated aminopeptidase (IRAP) are unknown.

- We demonstrate that IRAP contributes to the normal maternal cardiovascular adaptations to pregnancy and placental development in mice.

- Improved understanding of pregnancy-related cardiovascular dysfunction is an unmet need that will inform efforts to improve pregnancy outcomes in women.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

Open Access

CRediT Author Contribution

Sarah L. Walton: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing — original draft, Writing — review and editing. **Katrina M. Mirabito Colafella:** Investigation, Methodology, Writing — review and editing. **Aneesa Ansari:** Investigation, Writing — review and editing. **Siew Yeen Chai:** Conceptualization, Resources, Writing — review and editing. **Kate M. Denton:** Conceptualization, Formal analysis, Project administration, Resources, Supervision, Validation, Writing — original draft, Writing — review and editing.

Acknowledgements

Abbreviations

EVT, extravillous trophoblast; HR, heart rate; IRAP, insulin-regulated aminopeptidase; RAAS, renin–angiotensin–aldosterone system.

References

1 Chapman, A.B., Abraham, W.T., Zamudio, S., Coffin, C., Merouani, A., Young, D. et al. (1998) Temporal relationships between hormonal and hemodynamic changes in early human pregnancy. *Kid. Int.* **54**, 2056–2063, https://doi.org/10.1046/j.1523-1755.1998.00217.x

2 Lumbers, E.R., Delforce, S.J., Arthurs, A.L. and Pringle, K.G. (2019) Causes and consequences of the dysregulated maternal renin-angiotensin system in preeclampsia. *Front Endocrinol* **10**, 563, https://doi.org/10.3389/fendo.2019.00563

3 Irani, R.A. and Xia, Y. (2008) The functional role of the renin–angiotensin system in pregnancy and preeclampsia. *Placenta* **29**, 763–771, https://doi.org/10.1016/j.placenta.2008.06.011

4 Lew, R.A., Mustafa, T., Ye, S., McDowall, S.G., Chai, S.Y. and Albiston, A.L. (2003) Angiotensin at4 ligands are potent, competitive inhibitors of insulin regulated aminopeptidase (irap). *J. Neurochem.* **86**, 344–350, https://doi.org/10.1046/j.1471-4159.2003.01852.x

5 Nagasaka, T., Nomura, S., Okamura, M., Tsujimoto, M., Nakazato, H., Oiso, Y. et al. (1997) Immunohistochemical localization of placental leucine aminopeptidase/oxytocinase in normal human placental, fetal and adult tissues. *Reprod. Fertil. Dev.* **9**, 747–754, https://doi.org/10.1071/R97055

6 Nomura, M., Tsukahara, S., Ando, H., Katsumata, Y., Okada, M., Itakura, A. et al. (2002) Differential distribution of placental leucine aminopeptidase/oxytocinase and aminopeptidase a in human trophoblasts of normal placenta and complete hydatidiform mole. *Placenta* **23**, 631–639, https://doi.org/10.1053/plac.2002.0861

7 Williams, P., Mistry, H., Innes, B., Bulmer, J. and Pipkin, F.B. (2010) Expression of at1r, at2r and at4r and their roles in extravillous trophoblast invasion in the human. *Placenta* **31**, 448–455, https://doi.org/10.1016/j.placenta.2010.02.014

8 Ino, K., Kikkawa, F., Suzuki, T., Kajiyama, H., Shibata, K., Nomura, S. et al. (2003) Expression of placental leucine aminopeptidase and adipocyte-derived leucine aminopeptidase in human normal and malignant invasive trophoblastic cells. *Lab. Invest.* **83**, 1799–1809, https://doi.org/10.1097/01.LAB.0000101728.93907.75

9 Yamahara, N., Nomura, S., Suzuki, T., Itakura, A., Ito, M., Okamoto, T. et al. (2000) Placental leucine aminopeptidase/oxytocinase in maternal serum and placenta during normal pregnancy. *Life Sci.* **66**, 1401–1410, https://doi.org/10.1016/S0024-3205(00)00451-3

10 Ofner, L.D. and Hooper, N.M. (2002) Ectodomain shedding of cystinyl aminopeptidase from human placental membranes. *Placenta* **23**, 65–70, https://doi.org/10.1053/plac.2001.0751

11 Schrier, R.W. (2010) Systemic arterial vasodilation, vasopressin, and vasopressinase in pregnancy. *J. Am. Soc. Nephrol.* **21**, 570–572, https://doi.org/10.1681/ASN.2009060653

12 Nomura, S., Ito, T., Yamamoto, E., Sumigama, S., Iwase, A., Okada, M. et al. (2005) Gene regulation and physiological function of placental leucine aminopeptidase/oxytocinase during pregnancy. *Biochim. Biophys. Acta* **1751**, 19–25, https://doi.org/10.1016/j.bbapap.2005.04.006

13 Wallis, M.G., Lankford, M.F. and Keller, S.R. (2007) Vasopressin is a physiological substrate for the insulin-regulated aminopeptidase irap. *Am. J. Physiol. Endocrinol. Metab.* **293**, E1092–E1102, https://doi.org/10.1152/ajpendo.00440.2007

14 Matsumoto, H., Rogi, T., Yamashiro, K., Kodama, S., Tsuruoka, N., Hattori, A. et al. (2000) Characterization of a recombinant soluble form of human placental leucine aminopeptidase/oxytocinase expressed in chinese hamster ovary cells. *Eur. J. Biochem.* **267**, 46–52, https://doi.org/10.1046/j.1432-1327.2000.00949.x

15 Tsujimoto, M., Mizutani, S., Adachi, H., Kimura, M., Nakazato, H. and Tomoda, Y. (1992) Identification of human placental leucine aminopeptidase as oxytocinase. *Arch. Biochem. Biophys.* **292**, 388–392, https://doi.org/10.1016/0003-9861(92)90007-J

16 Cohen, M., Wuillemin, C., Chai, S.Y. and Bischof, P. (2008) Effects of ligands or substrate of insulin-regulated aminopeptidase (irap) on trophoblast invasion. *J. Peptide Sc.: An Off. Publication Eur. Peptide Soc.* **14**, 883–887, https://doi.org/10.1002/psc.1018

17 Kozaki, H., Itakura, A., Okamura, M., Ohno, Y., Wakai, K. and Mizutani, S. (2001) Maternal serum placental leucine aminopeptidase (p-lap)/oxytocinase and preterm delivery. *Int. J. Gynecol. Obstet.* **73**, 207–213, https://doi.org/10.1016/S0020-7292(01)00372-1

18 Landau, R., Laverrière, A., Bischof, P., Irion, O., Morales, M. and Cohen, M. (2010) Alteration of circulating placental leucine aminopeptidase (p-lap) activity in preeclampsia. *Neuroendocrinol. Lett.* **31**, 63–66

19 Lyall, F., Robson, S.C. and Bulmer, J.N. (2013) Spiral artery remodeling and trophoblast invasion in preeclampsia and fetal growth restriction. *Hypertension* **62**, 1046–1054, https://doi.org/10.1161/HYPERTENSIONAHA.113.01892

20 Albiston, A.L., Fernando, R.N., Yeatman, H.R., Burns, P., Ng, L., Daswani, D. et al. (2010) Gene knockout of insulin-regulated aminopeptidase: Loss of the specific binding site for angiotensin iv and age-related deficit in spatial memory. *Neurobiol. Learn. Mem.* **93**, 19–30, https://doi.org/10.1016/j.nlm.2009.07.011

21 Barsha, G., Denton, K.M. and Colafella, K.M.M. (2016) Sex-and age-related differences in arterial pressure and albuminuria in mice. *Biol. Sex Differ.* **7**, 57, https://doi.org/10.1186/s13293-016-0110-x

22 Gårdebjer, E.M., Cuffe, J.S.M., Pantaleon, M., Wlodek, M.E. and Moritz, K.M. (2014) Periconceptional alcohol consumption causes fetal growth restriction and increases glycogen accumulation in the late gestation rat placenta. *Placenta* **35**, 50–57, https://doi.org/10.1016/j.placenta.2013.10.008

23 Wilson, R.L., Leemaqz, S.Y., Goh, Z., McAninch, D., Jankovic-Karasoulos, T., Leghi, G.E. et al. (2017) Zinc is a critical regulator of placental morphogenesis and maternal hemodynamics during pregnancy in mice. *Sci. Rep.* **7**, 1–14, https://doi.org/10.1038/s41598-017-15085-2

24 Walton, S.L., Singh, R.R., Little, M.H., Bowles, J., Li, J. and Moritz, K.M. (2018) Prolonged prenatal hypoxia selectively disrupts collecting duct patterning and postnatal function in male mouse offspring. *J. Physiol.* **596**, 5873–5889, https://doi.org/10.1113/JP275918

25 Savu, O., Jurcuţ, R., Giuşcă, S., Mieghem, T.v., Gussi, I., Popescu, B.A. et al. (2012) Morphological and functional adaptation of the maternal heart during pregnancy. *Circ. Cardiovasc. Imaging* **5**, 289–297, https://doi.org/10.1161/CIRCIMAGING.111.970012

26 Gyselaers, W. and Thilaganathan, B. (2019) Preeclampsia: A gestational cardiorenal syndrome. *J. Physiol.* **597**, 4695–4714, https://doi.org/10.1113/JP274893

27 Ouzounian, J.G. and Elkayam, U. (2012) Physiologic changes during normal pregnancy and delivery. *Cardiol. Clin.* **30**, 317–329, https://doi.org/10.1016/j.ccl.2012.05.004

28 Ngene, N.C. and Moodley, J. (2019) Physiology of blood pressure relevant to managing hypertension in pregnancy. *J. Matern. Fetal Neonatal. Med.* **32**, 1368–1377, https://doi.org/10.1080/14767058.2017.1404569

29 Wong, A.Y.H., Kulandavelu, S., Whiteley, K.J., Qu, D., Langille, B.L. and Adamson, S.L. (2002) Maternal cardiovascular changes during pregnancy and postpartum in mice. *Am. J. Physiol. Heart Circ. Physiol.* **282**, H918–H925, https://doi.org/10.1152/ajpheart.00641.2001

30 Spradley, F.T. (2019) Sympathetic nervous system control of vascular function and blood pressure during pregnancy and preeclampsia. *J. Hypertens.* **37**, 476–487, https://doi.org/10.1097/HJH.0000000000001901

31 Yang, C.C.H., Chao, T.-C., Kuo, T.B.J., Yin, C.-S. and Chen, H.I. (2000) Preeclamptic pregnancy is associated with increased sympathetic and decreased parasympathetic control of hr. *Am. J. Physiol. Heart Circ. Physiol.* **278**, H1269–H1273, https://doi.org/10.1152/ajpheart.2000.278.4.H1269

32 de Haas, S., Ghossein-Doha, C., van Kuijk, S.M.J., van Drongelen, J. and Spaanderman, M.E.A. (2017) Physiological adaptation of maternal plasma volume during pregnancy: A systematic review and meta-analysis. *Ultrasound Obstet. Gynecol.* **49**, 177–187, https://doi.org/10.1002/uog.17360

33 Salas, S.P., Marshall, G., Gutiérrez, B.L. and Rosso, P. (2006) Time course of maternal plasma volume and hormonal changes in women with preeclampsia or fetal growth restriction. *Hypertension* **47**, 203–208, https://doi.org/10.1161/01.HYP.0000200042.64517.19

34 Cheung, K.L. and Lafayette, R.A. (2013) Renal physiology of pregnancy. *Adv. Chronic Kidney Dis.* **20**, 209–214, https://doi.org/10.1053/j.ackd.2013.01.012

35 Masuda, S., Hattori, A., Matsumoto, H., Miyazawa, S., Natori, Y., Mizutani, S. et al. (2003) Involvement of the v2 receptor in vasopressin-stimulated translocation of placental leucine aminopeptidase/oxytocinase in renal cells. *Eur. J. Biochem.* **270**, 1988–1994, https://doi.org/10.1046/j.1432-1033.2003.03570.x

36 Albiston, A.L., Yeatman, H.R., Pham, V., Fuller, S.J., Diwakarla, S., Fernando, R.N. et al. (2011) Distinct distribution of glut4 and insulin regulated aminopeptidase in the mouse kidney. *Regul. Pept.* **166**, 83–89, https://doi.org/10.1016/j.regpep.2010.09.003

37 Ishii, M., Naruse, K., Hattori, A., Tsujimoto, M., Ishiura, S., Numaguchi, Y. et al. (2009) Oxytocin hypersensitivity in pregnant p-lap deficient mice. *Life Sci.* **84**, 668–672, https://doi.org/10.1016/j.lfs.2009.02.018

38 Lumbers, E.R. (2020) The physiological roles of the renin-angiotensin aldosterone system and vasopressin in human pregnancy. *Maternal-Fetal Neonatal Endocrinol. Elsevier* 129–145, https://doi.org/10.1016/B978-0-12-814823-5.00010-6

39 Hofmeyr, G.J., Gülmezoglu, A.M. and Novikova, N. (2002) Maternal hydration for increasing amniotic fluid volume in oligohydramnios and normal amniotic fluid volume. *Cochrane Database Syst. Rev.*, https://doi.org/10.1002/14651858.CD000134

40 McKenzie, A.L., Muñoz, C.X., Ellis, L.A., Perrier, E.T., Guelinckx, I., Klein, A. et al. (2017) Urine color as an indicator of urine concentration in pregnant and lactating women. *Eur. J. Nutr.* **56**, 355–362, https://doi.org/10.1007/s00394-015-1085-9

41 Santillan, M.K., Santillan, D.A., Scroggins, S.M., Min, J.Y., Sandgren, J.A., Pearson, N.A. et al. (2014) Vasopressin in preeclampsia: A novel very early human pregnancy biomarker and clinically relevant mouse model. *Hypertension* **64**, 852–859, https://doi.org/10.1161/HYPERTENSIONAHA.114.03848

42 Pham, V., Burns, P., Albiston, A.L., Yeatman, H.R., Ng, L., Diwakarla, S. et al. (2009) Reproduction and maternal behavior in insulin-regulated aminopeptidase (irap) knockout mice. *Peptides* **30**, 1861–1865, https://doi.org/10.1016/j.peptides.2009.07.019

43 De Clercq, K., Lopez-Tello, J., Vriens, J. and Sferruzzi-Perri, A.N. (2020) Double-label immunohistochemistry to assess labyrinth structure of the mouse placenta with stereology. *Placenta* **94**, 44–47, https://doi.org/10.1016/j.placenta.2020.03.014

44 Woods, L., Perez-Garcia, V. and Hemberger, M. (2018) Regulation of placental development and its impact on fetal growth—new insights from mouse models. *Front. Endocrinol.* **9**, 570, https://doi.org/10.3389/fendo.2018.00570

45 Iwanaga, K., Nomura, S., Ito, T., Ikoma, Y., Yamamoto, E., Okada, M. et al. (2003) Placental leucine aminopeptidase/oxytocinase gene regulation by activator protein-2 in bewo cell model of human trophoblast differentiation. *FEBS Lett.* **552**, 120–124, https://doi.org/10.1016/S0014-5793(03)00897-4

46 Nakata, M., Nomura, S., Ikoma, Y., Sumigama, S., Shido, F., Ito, T. et al. (2004) Insulin stimulates placental leucine aminopeptidase/oxytocinase/insulin-regulated membrane aminopeptidase expression in bewo choriocarcinoma cells. *Regul. Pept.* **117**, 187–193, https://doi.org/10.1016/j.regpep.2003.10.015

47 Akison, L.K., Nitert, M.D., Clifton, V.L., Moritz, K.M. and Simmons, D.G. (2017) Alterations in placental glycogen deposition in complicated pregnancies: Current preclinical and clinical evidence. *Placenta* **54**, 52–58, https://doi.org/10.1016/j.placenta.2017.01.114

48 Tunster, S.J., Watson, E.D., Fowden, A.L. and Burton, G.J. (2020) Placental glycogen stores and fetal growth: Insights from genetic mouse models. *Reproduction* **159**, R213–R235, https://doi.org/10.1530/REP-20-0007

49 Fernández-Atucha, A., Izagirre, A., Fraile-Bermúdez, A.B., Kortajarena, M., Larrinaga, G., Martinez-Lage, P. et al. (2017) Sex differences in the aging pattern of renin–angiotensin system serum peptidases. *Biol. Sex Differ.* **8**, 5, https://doi.org/10.1186/s13293-017-0128-8

50 Clifton, V.L. (2010) Review: Sex and the human placenta: Mediating differential strategies of fetal growth and survival. *Placenta* **31**, S33–S39, https://doi.org/10.1016/j.placenta.2009.11.010

51 Kalisch-Smith, J.I., Simmons, D.G., Pantaleon, M. and Moritz, K.M. (2017) Sex differences in rat placental development: From pre-implantation to late gestation. *Biol. Sex Differ.* **8**, 17, https://doi.org/10.1186/s13293-017-0138-6