A sexually dimorphic murine model of IUGR induced by embryo transfer

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Abstract

Animal models are needed to develop interventions to prevent or treat intrauterine growth restriction (IUGR). Foetal growth rates and effects of *in utero* exposures differ between sexes, but little is known about sex-specific effects of increasing litter size. We established a murine IUGR model using pregnancies generated by multiple embryo transfers, and evaluated sex-specific responses to increasing litter size. CBAF1 embryos were collected at gestation day 0.5 (GD0.5) and 6, 8, 10 or 12 embryos were transferred into each uterine horn of pseudopregnant female CD1 mice ($n = 32$). Foetal and placental outcomes were measured at GD18.5. In the main experiment, fetuses were genotyped (*Sry*) for analysis of sex-specific outcomes. The number of implantation sites ($P = 0.033$) and litter size (number of fetuses, $P = 0.008$) correlated positively with the number of embryos transferred, while placental weight correlated negatively with litter size (both $P < 0.01$). The relationship between viable litter size and foetal weight differed between sexes (interaction $P = 0.002$), such that foetal weights of males ($P = 0.002$), but not females ($P = 0.233$), correlated negatively with litter size. Placental weight decreased with increasing litter size ($P < 0.001$) and was lower in females than males ($P = 0.020$). Our results suggest that male fetuses grow as fast as permitted by nutrient supply, whereas the female maintains placental reserve capacity. This strategy reflecting sex-specific gene expression is likely to place the male foetus at greater risk of death in the event of a 'second hit'.

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Introduction

Intrauterine growth restriction (IUGR), where a suboptimal foetal environment prevents the foetus from attaining its genetically determined growth potential, affects 6–12% of pregnancies in developed countries (Kramer 2003). Babies born from IUGR pregnancies often have a low birth weight (LBW, <2.5 kg), although this can also result from prematurity without IUGR (Gardosi *et al.* 2018). In developed countries, one of the most common causes of IUGR is placental insufficiency (Kramer 2003). This typically affects the foetus in the later stages of gestation (>32 weeks) and results in an asymmetric reduction in foetal biometric parameters like abdominal circumference, while the growth of the foetal brain is relatively preserved (Fleiss *et al.* 2019). LBW and IUGR are associated with increased risks of both neonatal complications, including stillbirth, and persistent impacts on health, including ~20–30% increased risks for cardiometabolic diseases (Barker 1994, Berends & Ozanne 2012, Gardosi *et al.* 2013,

Australian Institute of Health Welfare 2019). There is currently no cure or prevention for IUGR and robust animal models are needed to develop and test interventions.

In humans, the risk of LBW increases in multiple births; 5.2% of singletons, 55% of twins and 99% of higher-order multiples in Australia were LBW in 2017 (Australian Institute of Health Welfare 2019). In mice, as in other litter-bearing species such as pig, placental and foetal weights decrease as litter size increases (McLaren 1965, Gluckman *et al.* 1992, Gatford *et al.* 2004, Ishikawa *et al.* 2006). Seminal studies demonstrated restricted foetal growth due to maternal constraint in the context of cross-breeding of horses (Walton & Hammond 1938) and, embryo transfer experiments in horses (Tischner 1985, Allen *et al.* 2002) and pigs (Ashworth *et al.* 1990) that remove confounding effects of imprinting. More recently, the embryo transfer technique was also used in rabbits to demonstrate that foetal size is larger in smaller litters (Chavatte-Palmer *et al.* 2008). These studies clearly show that foetal

growth is limited by maternal size, despite evidence of some compensatory adaptations to placental size and structure (Allen *et al.* 2002). The mechanisms for constrained foetal growth due to increasing litter size is likely to differ somewhat from those seen in singleton pregnancies. However, larger litters are subject to both reduced placental growth and function and competition for maternal nutrients, since reducing litter size from twin to singleton post-implantation only partially restores foetal growth in sheep (Hancock *et al.* 2012).

Litter size increases as a greater number of embryos are transferred to pseudopregnant mice (Johnson *et al.* 1996), producing a range of litter sizes independent of these maternal confounders, although foetal and placental growth have not been reported in this model. Our primary aim was therefore to establish and characterise a model of variable foetal constraint due to increasing litter size induced through embryo transfer in mice. In human pregnancies, median weights of male foetuses are higher than female foetuses at all gestational ages up to term (Dobbins *et al.* 2012, Broere-Brown *et al.* 2016). Their rapid growth strategy is believed to put male infants at a higher risk of preterm birth and neonatal mortality compared to female infants, and foetal and placental responses to an adverse environment also differ between sexes in humans and experimental animal models (Clifton 2010, Gabory *et al.* 2013, Tarrade *et al.* 2015, Kalisch-Smith *et al.* 2017). Therefore, our secondary aim was to assess sex-specific responses to increasing litter size in our experimental model.

Materials and methods

Ethics and experimental design

Experimental procedures were approved by the University of Adelaide Animal Ethics Committee (M-2016-186) and carried

out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia 2013). Mice used in this study were housed at ~23°C with 12 h light:12 h darkness cycle (lights on 06:00 h), with access to water and meat-free rat and mouse diet *ad libitum* (14.0 MJ/kg, 20% protein, Speciality Feeds, Glen Forrest, Australia).

Animal experiments

Foetal and placental outcomes were assessed in recipient dams that received between 12 and 24 embryos to induce variable litter sizes (Fig. 1). Virgin female CBAF1 mice at 21- to 25-day-old were purchased from the University of Adelaide breeding colony. Mice were super-ovulated by intraperitoneal (i.p.) injection of pregnant mare's serum gonadotropin (Folligon, 5 IU; Intervet Australia, Victoria, Australia), followed 46–48 h later by an i.p. injection of human chorionic gonadotropin (hCG, Chorulon, 5 IU, Intervet Australia) to induce ovulation. These mice were then housed 1:1 with proven fertile CBAF1 males, and 1-cell embryos were collected by humanely killing the mouse and flushing the tract, 23 h after hCG injection. Embryos were vitrified, thawed and transferred to pseudopregnant CD1 females (Nakao *et al.* 1997), in the South Australian Health and Medical Research Institute (SAHMRI) facility. All transfers were conducted by the same operator.

To induce pseudopregnancy, mature (7- to 10-week-old) CD1 female mice from the SAHMRI breeding colony were mated at oestrus to vasectomised male CBAF1 mice by housing two females with a male overnight. The following morning, females with a vaginal plug were anaesthetised using gas anaesthesia (isoflurane, 2% O₂ at 2 L per minute) and analgesia (single dose of 0.1 mL Buprenorphine (Temgesic) administered subcutaneously at the rate of 0.05–0.1 mg/kg). To access each uterine horn, a small cut was made in the skin and muscle wall, and the upper part of the reproductive tract was gently exteriorised. Either 6, 8, 10 or 12 embryos were transferred to the ampulla of each uterine horn using a fine

Figure 1 Experimental design.

pipette, resulting in the transfer of a total of 12, 16, 20 and 24 embryos per dam, respectively ($n=8$ dams per group, $n=32$ dams in total). The reproductive tract was then returned to the abdominal cavity and the skin closed with surgical clips. Females were pair-housed after recovery and wound clips were removed 7–10 d post-surgery (Nakao *et al.* 1997). The transfer was noted as unsuccessful during the procedure in one horn of a mouse receiving six embryos per horn, and data from this animal was excluded from analyses of the relationship between the number of embryos transferred and litter size.

At GD17.5, 0.2 nmol/g body weight of IRDye 800CW 2-deoxyglucose (IRDye 800CW 2-DG) was injected via the tail vein of each pregnant mouse (Kaur *et al.* 2019), which were then returned to pair-housing (Fig. 1). Foetal uptake of IRDye 800CW 2-DG correlates positively with placental efficiency, and foetal growth parameters. However, the foetal fluorescence signal is similar for fluorophore conjugated to glucose or carboxylate (negative control), so should be interpreted as a measure of overall placental function and nutrient supply to the foetus in murine pregnancy, not as a marker of foetal glucose accumulation (Kaur *et al.* 2019).

Approximately 24 h later, at GD18.5 (Fig. 1), the mice were anaesthetised with isoflurane (5% induction, 1.5% maintenance, in medical air). Blood flow velocities of the uterine artery ($n=30$ dams) and from the umbilical arteries of at least two, and where possible, four foetuses per dam ($n=66$ male foetuses; $n=49$ female foetuses) were assessed *in vivo* using an ultrasound biomicroscope (Vevo 3100, VisualSonics®, ON, Canada) as described previously (Poudel *et al.* 2013, Care *et al.* 2015). These measurements were transabdominal, and therefore Doppler ultrasound of umbilical vessels could only be performed for foetuses located adjacent to the maternal ventral surface. Foetal location was marked on the skin of the anaesthetised dam using permanent texta during the ultrasound. At postmortem, conducted immediately after ultrasound of anaesthetised dams, the maternal abdomen wall was opened carefully without moving the uterus, and marks were made on the uterus to indicate the studied foetuses. The foetuses located at these positions were dissected and weighed before other foetuses to ensure accurate identification. Peak systolic velocity (PSV), end-diastolic velocity (EDV), time-averaged velocity (TAV) and heart rate averages were obtained from a minimum of three consecutive cardiac cycles. Resistance index ($RI=(PSV - EDV)/PSV$) and pulsatility index ($PI=(PSV - EDV)/TAV$) were calculated. Following Doppler imaging, dams were humanely killed by cervical dislocation, and foetuses and placentas were dissected and weighed. Placental and foetal weight ($n=157$ males; $n=141$ females) were measured for all pregnancies. Every alternate placenta per dam was fixed as described previously. Additional foetal size measures (abdominal circumference, crown to rump length, and head width) were made on all individual foetuses by a single researcher using cotton thread and Vernier callipers in all pregnancies after the initial 10 postmortems ($n=22$ dams; $n=105$ male foetuses; $n=100$ female foetuses). After completion of foetal size measures, anaesthetised foetuses were killed by decapitation, and foetuses and foetal tails were snap-frozen.

Determination of foetal sex

DNA was extracted from foetal tails using QuickExtract DNA Extraction Solution (Epicentre, Madison, Wisconsin, USA). PCR reactions for *Sry* were made up of 20 μ L mastermix containing 0.2 μ L Taq polymerase (Platinum™ Taq DNA Polymerase, Invitrogen), 10 μ L FailSafe™ PCR Enzyme and 2 $\times$ PreMix Buffer D (Epicentre) and 10 μ M *Sry* primers (forward: 5'-AACAACTGGGCTTTGCACATTG-3', reverse: 5'-GTTTATCAGGGTTTCTCTCTAGC-3' (final concentration 0.5 nM) and samples were run for 33 cycles (94°C for 1 min, 60°C for 1 min, 72°C for 72 s) followed by 9 min at 72°C on a GeneAmp® PCR system 9700 (Applied Biosystems). Male foetuses were determined by the presence of *Sry*. Female foetuses were defined by a negative *Sry* reaction, together with the presence of intact DNA, confirmed by a positive reaction for *Myogenin*, assessed using the same protocol and using forward and reverse primers for *Myogenin* (forward: 5'-TTACGTCCATCGTGGACAGC-3', reverse: 5'-TGGGCTGGGTGTTAGTCTTA-3') (McClive & Sinclair 2001). DNA was re-extracted and PCR repeated for any sample where *Myogenin* was not detected. Outcomes of the PCR were validated by gel electrophoresis on a 2% agarose gel.

Placental morphology and function

Frozen foetuses were homogenised and fluorescence-imaged at 800 nm using the Odyssey CLx Infrared Imaging System (Kaur *et al.* 2019). An index of placental transfer of IRDye 800CW 2-DG, which correlates positively with placental efficiency and negatively with umbilical cord resistance index, was calculated as total foetal fluorescence at 800 nm divided by placental weight ($n=154$ males, $n=140$ females, Kaur *et al.* 2019).

Bisected placentas ($n=2-6$ per dam) were fixed in 4% paraformaldehyde (PFA), washed in 1 $\times$ PBS over a 48 h period and stored in 70% ethanol prior to being paraffin embedded. Full-faced mid-sagittal placental sections cut to 5 μ m thickness were stained with Masson's Trichrome following standard protocols (Roberts *et al.* 2003). Areas of junctional zone and placental labyrinth for each placental section were visualised and measured with NDP.view 2 software (Hamamatsu Photonics, Shizuoka, Japan). Total cross-sectional area and the proportion of junctional zone to placental labyrinth were calculated for each placenta.

Statistical analysis

Relationships between numbers of embryos transferred and numbers of implantation sites and foetuses in each experiment were assessed by Pearson's correlation. Effects of foetal sex (fixed factor) and litter size (covariate) on maternal, foetal and placental outcomes were analysed using the mixed models procedure in SPSS (version 24.0, IBM) and including dam as a random variable to account for the common maternal environment. Foetal dye uptake data were not normally distributed and was log-transformed for analysis. Previous studies have reported that murine foetuses at either

Figure 2 The numbers of viable implantation sites (A) correlated positively with the number of embryos transferred in mice. The total number of viable foetuses in the litter (B) also increased with the number of embryos transferred. P and R values are derived from Pearson's correlation analysis; $n = 31$ litters).

end of the uterine horns are heavier than those located near the middle of the uterine horns (McLaren & Michie 1960). In preliminary analyses we therefore also added foetal position within the uterine horn as a covariate; this was not significant and was removed from final models. Because effects of litter size on foetal weight differed between males and females, foetal morphometric data were also analysed separately within each sex. Relationships between foetal dye uptake and umbilical blood flow parameters and foetal weight and size were assessed by Pearson's Correlation analysis using data for each individual foetus. Figures show points for individual litters or foetuses/placentae, except where otherwise indicated, and $P < 0.05$ was considered significant for all analyses.

Results

Increased litter size induces sex-specific foetal IUGR

Both the number of implantation sites ($R = 0.38$, $P = 0.033$, Fig. 2A) and litter size ($R = 0.47$, $P = 0.008$, Fig. 2B) correlated positively with the number of embryos transferred.

The relationship between litter size and foetal weight differed between males and females (interaction $P = 0.002$), such that foetal weight was negatively correlated with litter size in male ($P < 0.001$), but not female ($P = 0.211$) foetuses (Fig. 3A). In contrast, abdominal circumference, foetal head width and crown to rump length did not differ between sexes (all $P > 0.05$). As litter size increased, foetal abdominal circumference decreased overall ($P = 0.003$, Fig. 3B), within males ($P = 0.003$) and within females ($P = 0.035$). Foetal head width and crown to rump length were not related to litter size overall ($P > 0.1$, Fig. 3C and D, respectively), or within each sex. Markers of IUGR were observed in foetuses from larger litters. Specifically, as litter size increased, foetal weight to crown-rump length ratio decreased overall ($P = 0.012$, Fig. 3E), and in males ($P = 0.007$) but not females. Similarly, with increasing litter size foetal head width to abdominal circumference tended

to increase overall ($P = 0.063$, Fig. 3F), and increased in males ($P = 0.026$) but not females.

Increased litter size impairs placental size and function similarly in both sexes

Placental weight correlated negatively with litter size ($P < 0.001$) and was lower in females than males ($P = 0.022$), but the effect of litter size on placental weight did not differ between sexes (Fig. 4A). Placental efficiency tended to correlate positively with litter size ($P = 0.055$), and the effect of litter size on placental efficiency was similar in males and females ($P > 0.4$, Fig. 4B). Similarly, foetal dye uptake per gram of placenta correlated positively with litter size ($P = 0.005$), and this relationship was similar between sexes ($P > 0.0.9$, Fig. 4C). Placental labyrinth ($P = 0.028$) and junctional zone ($P = 0.017$) areas correlated negatively with litter size, regardless of foetal sex (interactions: all $P > 0.4$) and did not differ between male and female placentas (all $P > 0.2$, Fig. 4D and E, respectively). The ratio of junctional to labyrinth zone areas was unaffected by litter size or sex (each $P > 0.7$, Fig. 4F). Pulsatility

Figure 3 Effects of litter size on foetal weight differed between sexes in mice. Weights of males, but not females, correlated negatively with litter size (A). Abdominal circumference (B) decreased with litter size but crown to rump length (C) and head width (D) were unchanged, regardless of foetal sex. With increasing litter size, all foetuses were thinner (E) with evidence of brain sparing (F). P values are derived from mixed model analyses of individual foetal data (weight $n = 298$, other size measures $n = 205$). Symbols show data from the individual male (grey triangles) and female (open circles) foetuses. Regression lines show correlations for combined sexes (black solid lines for significant correlations, grey solid lines for non-significant correlations); where sex or sex $\times$ litter size interactions were significant, correlations are shown separately for male (dotted line) and female (dashed line) foetuses.

Figure 4 Placental weight (A; $n = 298$) decreased with increasing litter size and was lower in females (open circles) compared to their male littermates (triangles), but effects of litter size did not differ between sexes in mice. Placental efficiency (foetal:placental weight; B; $n = 298$) and perfusion (C; $n = 295$) increased while both junctional zone (D; $n = 108$) and labyrinth zone (E; $n = 108$) areas decreased with litter size, irrespective of foetal sex. Ratio of junctional zone to labyrinth zone area (F; $n = 108$) was not affected by litter size or foetal sex. P values are derived from mixed model analyses of individual placental data, and data for foetal fluorophore uptake per gram placenta was log-transformed for analysis but non-transformed data are presented for ease of interpretation. Symbols show data from individual (A, B, D, E, F) or within sex litter averages (C) for male (grey triangles) and female (open circles) foetuses. Regression lines show correlations for combined sexes (black solid lines for significant correlations, grey solid lines for non-significant correlations); where sex or sex $\times$ litter size interactions were significant, correlations are shown separately for male (dotted line) and female (dashed line) foetuses.

and resistance indices for the umbilical artery did not change with litter size ($P > 0.70$ for both). Interestingly, the pulsatility index in the umbilical artery was lower in female than male foetuses ($P = 0.043$, Fig. 5B), with a similar trend for resistance index ($P = 0.054$). Uterine artery resistance and pulsatility indices did not correlate with litter size ($P > 0.05$, data not shown).

Discussion

We have shown that litter size increases linearly as more embryos are transferred to recipient female mice, at least up to a total of 24 embryos. Increasing litter size in this model induces an IUGR phenotype, with thinner foetuses and evidence of brain sparing near term. Furthermore, we have shown for the first time that foetal responses to increasing litter size are sex-specific, with constrained body weight, thinness and brain sparing evident in male but not female foetuses

Figure 5 Umbilical artery resistance (A) and pulsatility (B) indices from male (grey bars; $n = 66$) and female (white bars; $n = 49$) mouse foetuses at GD18.5. Data are represented as mean $\pm$ S.E.M. P values are derived from obtained from mixed model analysis of individual umbilical artery measurements.

as litter size increased. Male placentas were heavier than female placentas, but the effects of increasing litter size on placental weight and function did not differ between sexes. We have therefore developed an IUGR model independent of maternal confounders that affect ovulation rate (including breed or size), which can now be utilised to evaluate interventions.

In this present study, we report for the first time that increased litter size achieved by increasing the number of embryos transferred in mice, is associated with increasing degree of foetal and placental weight reduction near term, independent of maternal confounding factors. This adds to previous evidence that the litter size of mice could be nearly doubled by transferring the increasing number of zygotes into recipient mice, although foetal and placental outcomes were not characterised in this study (Johnson *et al.* 1996). Also consistent with our findings, Gluckman *et al.* reported a negative relationship between litter size and foetal growth in mice (Gluckman *et al.* 1992). In contrast to the present study, these authors transferred similar numbers of embryos into each dam, rather than generating a range of litter size by differential transfer, and the range of litter size that they observed, therefore, reflect maternal factors that alter implantation rate. Consistent with the results of Johnson *et al.*, who transferred up to 25 embryos per dam (Johnson *et al.* 1996), we did not see any evidence of increasing foetal deaths as we transferred more embryos into each uterine horn. This implicates variable implantation rate as the cause of variable litter sizes amongst dams that received the same numbers of embryos within the present study. We predict that foetal deaths might increase if the number of embryos was increased further to exceed the natural litter size range of the recipient strain, resulting in more resorptions, but this requires further experimentation.

Our findings of restricted foetal growth with the increasing litter size in mice are consistent with observations in non-litter-bearing species like humans and sheep, where the occurrence of multiple gestations reduces foetal growth rate relative to that of singletons (Rattray *et al.* 1974, Koong *et al.* 1975, Iffy *et al.* 1983). The foetal growth trajectory of twins diverges from that of singletons around 8 weeks in humans, and a slower foetal growth in twins persists in late gestation in both humans and sheep (Rattray *et al.* 1974, Koong *et al.* 1975, Leveno *et al.* 1979, Iffy *et al.* 1983). Similar to our results, foetal length in late gestation decreased with increasing foetal number in rabbit pregnancies generated by embryo transfer, although within a limited range of litter sizes that were all below those of natural pregnancies (8–10), since a maximum of 6 embryos were transferred to each recipient (Chavatte-Palmer *et al.* 2008). In the rabbit, the negative relationship between numbers of embryos transferred and foetal size measures was detectable by ultrasound from the last third of pregnancy (Chavatte-Palmer *et al.* 2008). Given the larger litter sizes in our mouse model, relative to those of naturally bred dams, we expect that relationships between litter size and foetal growth would emerge earlier in pregnancy, but testing this hypothesis would require additional studies. Transfer of limited embryo numbers may explain the lack of relationship between litter size and placental length in this rabbit study, since low litter sizes would have resulted in limited competition for implantation area (Blasco *et al.* 1994). In contrast, placental weight and litter size correlated negatively in embryo transfer murine pregnancies in both the present study and that of Gluckman *et al.*, where larger ranges in litter size were generated (Gluckman *et al.* 1992). Reduced birthweight in multiple pregnancies is similarly accompanied by reduced placental weight in humans (Bleker *et al.* 1979), and sheep (Vatnick *et al.* 1991, Gootwine *et al.* 2007).

A particularly interesting and novel finding of the present study was that the effect of an increasing litter size on foetal growth was sex-specific. In smaller litters, male foetuses weighed more than their female littermates in late gestation. It is now well established in humans, sheep and rodents that the average weights of male foetuses are heavier than females at an equivalent gestational age (Kent 1992, Chahoud & Paumgarten 2005, Dobbins *et al.* 2012, Broere-Brown *et al.* 2016). In the present study, as litter size increased, the weight of male foetuses decreased while weights of female foetuses remained stable. Other markers of asymmetric growth restriction, that is, weight:crown-rump length (thinness) and foetal head width:abdominal circumference (brain sparing), were also evident with increasing litter size in male foetuses but not their female littermates. Two hypotheses may explain this sex-specific relationship. First, growth strategies may differ between sexes, such that females maintain a 'placental reserve' by not growing to their full genetic capacity, even in smaller

litters where competition for nutrients is likely within a normal range. Conversely, a maximal growth strategy in males may mean their growth is limited by the available nutrient supply when either faced with an external insult or limitations to supply. Although we did not observe sex differences in foetal:placental weight ratio, which is considered a marker of placental efficiency (Hayward *et al.* 2016), we did not measure placental nutrient transport in our late gestation pregnancies, which would provide a direct, stage-specific measure of placental function. Evaluating these outcomes in male and female placentas under 'normal' conditions and in response to an *in utero* challenge might also provide useful insight. This hypothesis of a female 'placental reserve' capacity is also consistent with reports that although IUGR is more prevalent in females (Clifton *et al.* 2009), males have higher rates of spontaneous preterm birth and perinatal death if there is a perturbation in pregnancy (Ravelli *et al.* 1999, Ozaki *et al.* 2001, Ingemarsson 2003, Vatten & Skjærven 2004, Murphy *et al.* 2005, Eriksson *et al.* 2010). Secondly, female foetuses (and placentas) may be better able to adapt to an *in utero* environment where competition for nutrients is high, in order to maintain growth. This is supported by human and animal studies where expression of more genes alters in response to maternal undernutrition in female placentas compared to male placentas (Tarrade *et al.* 2015). These changes in placental gene expression likely act as a buffer to protect the female against *in utero* perturbations and possibly reduce the risk of adult-onset of diseases (Mao *et al.* 2010).

In addition to reduced foetal and placental weights, we found that increasing litter size in mice induced asymmetric foetal growth restriction in a sex-specific manner. Male foetuses in larger litters were thinner and showed evidence of brain sparing, regardless of sex. Skeletal growth, measured as crown-rump length, was not reduced with increasing litter size in the present study, suggesting that restriction in multi-foetal litters occurs later in gestation, after the spine develops (Bryan & Hindmarsh 2006, Briana *et al.* 2008). Similarly, a previous study in humans reported no differences in skeletal mass between asymmetrically growth restricted and AGA foetuses and neonates (Briana *et al.* 2008). This could be in part due to compensatory mechanisms in IUGR which favour the formation of mineralised bone, such as the downregulation of Dickkopf-1 (DKK-1), a protein involved in the inhibition of osteoblast differentiation (Briana *et al.* 2012). However, the effects of IUGR become apparent during adulthood, as growth-restricted individuals are predisposed to reduced peak skeletal size and mineralisation in later life (Briana *et al.* 2008). In humans, asymmetrical foetal growth restriction is also common in twins (Leveno *et al.* 1979, Iffy *et al.* 1983, Hirsch *et al.* 2020) and characteristic of IUGR foetuses (Dashe *et al.* 2000). This growth pattern is often associated with placental dysfunction,

which impairs oxygen and nutrient supply to the foetus, especially during late gestation when absolute foetal growth is at its fastest (Regnault 2003). In these foetuses, cardiac output is redirected towards maintaining growth of vital organs like the brain and heart, further impairing the growth of other organs including the liver and kidneys, contributing to the disproportionate foetal growth (Al-Ghazali *et al.* 1989). Structural and functional changes in the organs that accompany asymmetric growth restriction underlie adverse health outcomes that emerge in adolescent and adult life, including arterial hypertension and chronic renal failure (Leon *et al.* 1998, Ravelli *et al.* 1999, Alexander 2003, Briana *et al.* 2008, 2012). Asymmetric foetal growth is also observed in a number of animal models of IUGR, including maternal undernutrition in rabbits, mid-late gestation uterine artery ligation in rats, and reduction of placental exchange surface (through endometrial caruncle removal or repeated uteroplacental embolisation in sheep), which likewise restrict rapid foetal growth in late gestation (Wigglesworth 1964, De Blasio *et al.* 2007, López-Tello *et al.* 2015).

An additional major finding of the present study was that sex-specific relationships between litter size and foetal growth were not explained by sex-specific changes in placental size or function. The overall increase in placental efficiency and function as litter size increased in our study, is likely an adaptation aimed at optimising or maintaining foetal growth in the face of limited nutrient supply (Coan *et al.* 2008, Fowden *et al.* 2009, Sandovici *et al.* 2012, Wallace *et al.* 2013). Although in the present study placental size, efficiency and dye transfer (a marker of perfusion) increased similarly in males and females, it is possible that transport of specific nutrients might be differentially affected and hence explain the sex-specific constraint of foetal growth in large litters. Foetal susceptibility to adverse outcomes in a number of pregnancy complications appears to be sex-dependent (Vatten & Skjærven 2004) and is largely orchestrated by the placenta, which is genetically identical to the foetus (Roberts 2010). A number of different human and animal studies show that placentas may undergo sex-specific adaptations to the same *in utero* stressors (Kalisch-Smith *et al.* 2017). In males, placental adaptations appear to promote continued growth while in females, these adaptations are focussed on promoting placental development and foetal survival (Murphy *et al.* 2003). Although umbilical blood flow measures were not related to litter size, we did see some evidence of greater uteroplacental resistance in male compared to female foetuses near term. Although we were unable to assess placental microstructure in the present study, it has been suggested that greater vascular resistance at term in mice may reflect the decreased surface area of the maternal blood spaces in the labyrinth (Coan *et al.* 2004, Linask *et al.* 2014). Decreased area for exchange might therefore contribute to foetal growth restriction in

male foetuses. Variable structural adaptations, including increased foetal villi length, placental surface density and volume fraction have been reported in inter-breed embryo transfer pregnancies in horses, which may partially compensate for decreased placental weight and enable partial maintenance of foetal growth in the face of maternal constraint (Allen *et al.* 2002, Robles *et al.* 2018). Further studies are needed to characterise placental adaptations to variable litter size within our IUGR model.

An important strength of our study is the analysis by foetal sex, shown here to be an important determinant of foetal growth responses to the variable maternal constraint. An additional strength of our model is the removal of maternal factors that occur in studies of natural variation in litter size. In naturally conceived pregnancies, the spontaneous variation in litter size is dependent on maternal factors that determine ovulation rate, which increases with maternal weight, parity and age (Eckstein & McKeown 1955, Land 1970, French *et al.* 1979, Norman & Bruce 1979, Zamenhof & van Marthens 1984, Rödel *et al.* 2004). Our approach of transferring variable numbers of embryos removes factors that affect ovulation rate as confounders. This does, however, require the additional periconceptional exposures of super-ovulation, embryo culture and vitrification, which may also affect foetal development (Weinerman *et al.* 2017). Whether these effects differ with litter size or between sexes is, unknown, but we suggest the addition of a naturally mated control group that share embryonic and maternal genetics as a reference group within future experiments.

In summary, we have generated a murine model of IUGR reflective of multiple birth, with sex-specific foetal outcomes. Explanations for this must lie in the genetic differences between males and females conferred by the sex chromosomes and resultant hormonal differences. This model can be used to understand the mechanisms associated with IUGR induced by multifetal pregnancies, as well as to test possible interventions in future studies. Such analyses could compare the slope of the relationships between foetal and placental outcomes and litter size, as used here to compare sex-specific correlations, or could compare outcomes in large and small litters specifically. We suggest also, that potential therapies identified in the mouse model should be validated in other models prior to translation in humans, given the potential differences in foetal demand and placental signalling between twins or triplets in humans compared to higher-order multiples in mice. The availability of animal models of variable foetal constraint in which to evaluate interventions has substantial clinical relevance, given the substantial increases in the risks of perinatal death and lifelong adverse effects on health in IUGR infants and the current lack of effective treatments or preventative therapies.

Declaration of interest

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

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Author contribution statement

H K, A S C, R L W, B S M, C T R and K L G conceived and designed the experiments. H K, A S C, R L W, S G P and K L G were involved in the acquisition of data. H K, A S C, R L W, B S M, C T R and K L G analysed and interpreted the data. H K and K L G drafted the article. All authors critically revised and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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