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Review: Understanding the role of androgens and placental AR variants: Insight into steroid-dependent fetal-placental growth and development

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ABSTRACT

The role of steroids throughout pregnancy and their effect on placental physiology is well established, especially for estrogens, progestogens, and glucocorticoids; however, the role of androgens – particularly within the context of placental physiology – remains largely unexplored. Androgens are often defined as the male sex-steroids and are fundamental for the defeminisation and masculinisation of male fetuses. Therefore, the placenta may adapt to these steroids in a sex-specific manner, with males being more receptive to changes in these steroids concentrations, when compared with females; however, their involvement in female intrauterine development has been investigated in several studies and may suggest females have a level of responsiveness to these steroids. While the former may be true, studies have reported sex-specific differences in the expression and activity of factors involved in androgen biosynthesis and bioavailability, with males consistently demonstrating greater degrees of altered protein and gene expression when compared with females. Understanding the placental androgen axis is essential as many pregnancy comorbidities are associated with elevated concentrations of androgens and perturbed intrauterine development or growth. Indeed, it appears that specific pathophysiologies of pregnancy can modulate the activity of key factors involved in the placental androgen axis and this may contribute to the etiology of sex-specific developmental outcomes from certain pregnancy complications. This review will provide insight into what is currently known regarding androgen signalling and the human placenta, and how this complex system may regulate sex-specific growth and developmental outcomes in normal and adverse pregnancies.

1. Androgens and pregnancy

Throughout gestation, the levels of maternal circulating androgens including testosterone, dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), and androstenedione increase with concentrations increasing three-fold by the third trimester when compared to non-pregnant levels [1]. There are sex differences in the concentrations of androgens with cord blood testosterone concentrations being higher in males than females in normal pregnancies [2,3]. Studies further demonstrate that pregnancy comorbidities such as polycystic ovarian syndrome (PCOS), preeclampsia and gestational diabetes mellitus (GDM) are associated with an increase in the concentration of these sex-steroids [4–8]. The synthesis of androgens is dependent on the bioavailability of pregnenolone, an androgen precursor which is metabolised from cholesterol via the cholesterol side-chain cleavage enzyme, CYP11A1 (Fig. 1), in the mitochondria of select cell types, one of which is the placental trophoblast [9–11]. Within the placenta, progesterone is readily synthesised from pregnenolone via hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1) (Fig. 1), and can function as an androgen precursor via its conversion to 17α-Hydroxyprogesterone [12]. Interestingly, dysregulation of these initial enzymes involved in the biosynthesis of androgens has been identified in the placenta of specific pregnancy comorbidities.

Studies investigating placentae from preecamptic patients have demonstrated dysregulation of CYP11A1 and HSD3B1. Hogg et al. [13] observed significant hypomethylation of steroidogenic genes, including *CYP11A1* and *HSD3B1*, in the placenta of early and late onset preeclampsia, however the authors noted this observed hypomethylation may not be associated with changes in gene expression, and functional characterisation studies are still required. More recently, a study examining CYP11A1 function reported an increase in its protein expression in placentae of preeclamptic patients, when compared with control placentae [14]. The study also reported *in vitro* and *in vivo* CYP11A1-overexpression resulted in increased expression of autophagy markers including LC3-II, Beclin1, P62 and PINK-1, perturbed trophoblast invasion, and preeclamptic-like symptoms that were attenuated by the

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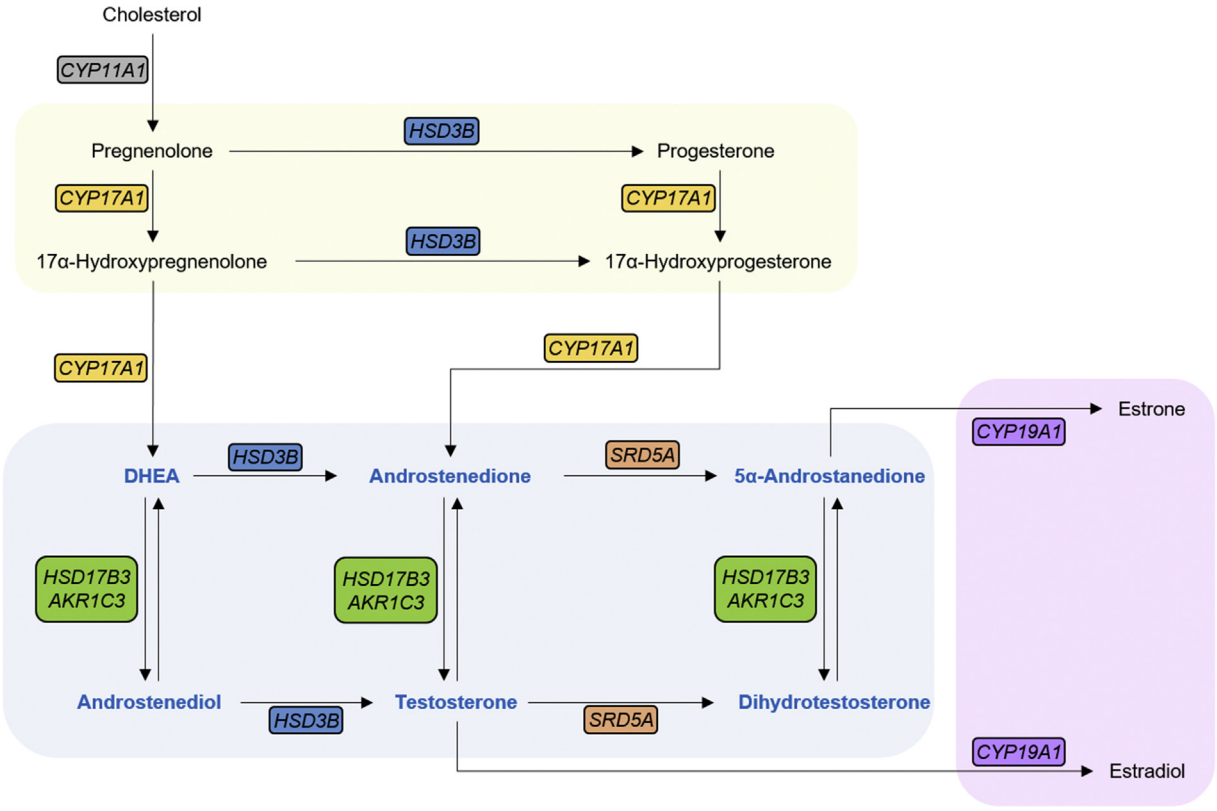

Fig. 1. – Androgens are synthesised via a cascade of enzymatic reactions. The first reaction in the process of androgen synthesis involves cleavage of the cholesterol side-chain via the cholesterol side-chain cleavage enzyme (CYP11A1). The resulting pregnenolone is either converted to progesterone or 17α-hydroxypregnenolone via 3β-hydroxystyeroid dehydrogenase (HSD3B) or cytochrome P450 17A1 (CYP17A1), respectively. Both progesterone and 17α-hydroxypregnenolone are then converted to 17α-hydroxyprogesterone via CYP17A1 or HSD3B, respectively, however 17α-hydroxypregnenolone can be directly metabolised to Dehydroepiandrosterone (DHEA) via CYP17A1. Androstenedione is synthesised via HSD3B's oxidation of DHEA or via hydroxylation of 17α-hydroxyprogesterone: this androgen derivative is then reduced to 5α-androstanedione via 5α-reductase (SRD5A). DHEA, androstenedione, and 5α-androstanedione can be converted to androstenediol, testosterone, and dihydrotestosterone, respectively, via 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3) or 17β-hydroxysteroid dehydrogenase type 5 (AKR1C3). Testosterone itself can also be synthesised via the oxidation of androstenediol, and this well-known androgen can then be reduced to dihydrotestosterone via SRD5A. The androgens testosterone and 5α-Androstanedione can further be metabolised to the estrogens estradiol and estrone, respectively, via aromatase (CYP19A1).

androgen receptor (AR) antagonist, flutamide. Collectively, these findings would suggest aberrant expression of steroidogenic enzymes can disrupt placental homeostasis, which may compromise placentation and fetal growth and development, and highlights the importance of androgen biosynthesis regulation throughout pregnancy.

Numerous proteins involved in androgen biosynthesis and signalling have been identified in the human placenta, and some studies would suggest that the expression and activity of these proteins may be altered by fetal-placental sex [4,15–17]. Under normal conditions, placental synthesised androgens are converted to estrogens by placental aromatase (CYP19A1) (Fig. 1). There are limited studies which have examined how placental sex or the presence of a pregnancy complication affects this process; however, one study examining the sex-specific dysregulation of androgen biosynthesis from placentae of preeclamptic pregnancies demonstrated decreased expression of CYP19A1 protein and mRNA in males, when compared to females [4]. Interestingly, female placentae of preeclamptic pregnancies had increased expression of CYP19A1 compared to female placentae from an uncomplicated pregnancy. Although the activity of aromatase was not measured, these findings, in conjunction with a reported increase in androgen concentrations in patients with preeclampsia and a male fetus suggest that in the presence of a pregnancy complication, males may prioritise androgen bioavailability by suppressing aromatase expression. Under similar adverse conditions, females prioritise estrogen bioavailability by upregulating the expression of aromatase. Likewise, a study conducted by Maliqueo et al. [17] reported reduced aromatase activity in placentae from patients diagnosed with PCOS, when compared to controls. This study, however, did not account for placental sex: rather, the study reported lower androstenedione and higher estriol concentrations in the cord blood of female newborns of patients with PCOS when compared to control, however the exact source of these steroids' biosynthesis remains unclear. Taken together, the two studies would indicate there are sex-specific alterations of androgen metabolism in the presence of a maternal complication, with an increase in placental aromatase activity in females and a decrease in males resulting in a rise in estrogen or androgen concentrations, respectively.

Androgen precursors that are not converted to estrogens are metabolised into the potent androgens testosterone and DHT. Protein and mRNA expression of cytochrome P450 17A1 (CYP17A1), an enzyme involved in androgen metabolism, has been detected in primary human trophoblast cells and the human trophoblast cell line JEG-3 [15]. These cells were able to generate testosterone *de novo*, however placental sex was not accounted for, which leaves questions surrounding placental sex and testosterone synthesis unanswered. Previous findings from our group have demonstrated male placentae at term have increased expression of 5α-reductase (SRD5A) compared to females [16]. This enzyme is involved in reducing testosterone to DHT (Fig. 1), a potent androgen derivative with a higher binding affinity for the androgen receptor AR than testosterone [18] and suggests that not only is the placenta a source of androgens, but that this organ may contribute to increased activity of the androgen axis in a sex-specific manner.

Current data suggests there are concentration differences in

androgens between the sexes in pregnancy and they are essential for sex determination, sex differences in fetal growth and possibly important in conferring sex-specific differences of the fetal-placental response to pregnancy complications or perturbations. Therefore, understanding i) the role androgens have in male and female intrauterine development ii) the role the placenta has in mediating these processes and iii) how pregnancy complications and perturbations of the placental androgen axis affects critical periods of growth and development are of particular interest.

2. Androgens: role in fetal growth and development

Androgens are essential for defeminisation (Müllerian ducts regression) and masculinisation (differentiation of Wollfian ducts to the epididymis, vasa deferentia, and the seminal vesicles) of the male fetus (for a review, see Rey et al. [19]). Interestingly, perturbations to androgen signalling via various mutations in the AR gene results in 46,XY karyotyped neonates presenting with female-like phenotypes [20,21]. These 46,XY neonates, who are clinically diagnosed as having partial or complete androgen insensitivity syndrome (AIS), were reported to have significantly reduced birthweight adjusted for gestational age when compared to a control male population [21]. Studies using global ARKO mouse models show similar findings, with males displaying infertility and a hyperfeminised phenotype absent of male reproductive organs, except testes [22]. It appears androgens and their receptor are fundamental for appropriate male growth and development *in utero* and any perturbation to this signalling pathway results in adverse outcomes.

Recent studies have demonstrated an involvement for androgens in female intrauterine development. Inhibition of androgen action via the antiandrogen flutamide resulted in perturbed folliculogenesis [23]. Knapczyk-Stwora et al. [23] reported altered expression of key targets involved in this process including *IGF-1, IGF-1R, c-KIT* and *KL*. A similar study using global and ovarian granulosa cell-specific AR knockout mouse models demonstrated, both *in vitro* and *in* vivo, that androgens at a physiological concentration attenuate follicular atresia via miR-125b and enhance follicle-stimulating hormone (FSH) receptor expression, which further increases FSH-mediated follicle growth and development [24].

Although androgens are important for female development, excessive levels present risks for female fetuses and the mother. Placental aromatase insufficiency has been shown to result in virilisation of female fetuses, due to the excessive levels of androgens in both maternal and fetal circulations [25–27]. Further, intrauterine exposure to high androgen levels is thought to be a contributing factor to the development of diseases later in life such as PCOS, metabolic disorders, and cardiovascular disease (CVD) [28–30]. The placenta is thought to mitigate excessive intrauterine androgen exposure of the female fetus through aromatase activity [31]. Certain pregnancy comorbidities, however, may detrimentally alter the expression and activity of androgen-specific placental enzymes and adversely affect female development. For this reason, understanding how pregnancy comorbidities affect key enzymes involved in androgen metabolism and biosynthesis needs further investigation. It appears that the dynamic state of androgen biosynthesis and bioavailability is fundamental for the appropriate development of female fetuses, and any perturbations to these processes can have adverse developmental outcomes and contribute to disease risks in adulthood.

It is unclear how androgens affect intrauterine growth outcomes. Human population studies have demonstrated small for gestational age (SGA) male neonates have significantly reduced levels of testosterone when compared to appropriate for gestational age (AGA) neonates [32]. Similarly, Carlsen et al. [33] demonstrated negative associations between testosterone levels at 17 and 33 weeks' gestation and birthweight (g) in female neonates. In contrast, Voegtline et al. [34] reported reduced male birthweight, but increased postnatal weight gain in pregnancies with high maternal plasma testosterone concentrations when compared with low testosterone. The same study reported increased female birthweight in the high testosterone group compared to the low testosterone group, but no change in postnatal weight gain. These discrepancies between studies are likely products of study design, which highlights the necessity for additional research investigating associations between androgen levels and birthweight outcomes, especially when considering a specific pregnancy comorbidity.

Sex differences in human fetal growth in pregnancy complications may be due to altered androgen levels. Our group has previously reported sex-specific birthweight outcomes from pregnancies complicated by mild preeclampsia [35]. Specifically, female neonates from pre-eclamptic pregnancies had significantly reduced birthweight centile (BWC) when compared to neonates from healthy control groups. In contrast, male BWC remained unaltered between normotensive and pre-eclamptic groups. Given previous studies report increased testosterone concentrations in pregnancies complicated by pre-eclampsia [5,36], it is possible that androgens have a role in regulating the differential intrauterine growth response between the sexes. However, further studies are required to substantiate this possibility.

In vivo animal studies have demonstrated that high levels of androgens during gestation can lead to IUGR-like outcomes, reduced fetal viability, and reduced litter size [37–41]. Cleys et al. [38] reported that the average birthweight of female offspring of testosterone propionate (TP) treated ewe was significantly reduced compared to controls, and that no difference in male birthweights were observed between treatment groups. These findings are in agreement with previous research conducted by Beckett et al. [37], which identified consistently lower birthweights of female offspring born to aromatisable (testosterone) and non-aromatisable (DHT) androgen treated ewes. In contrast, Sathishkumar et al. [41] reported, in a rodent model, that increased maternal testosterone concentrations suppressed fetal growth indirectly by affecting placental amino acid delivery to the fetus. Prenatal TP exposure significantly reduced *slc38a4* mRNA and protein expression in both male and female rat placentae and was associated with reduced placental amino acid transport capacity with a greater effect observed in males. Based on both human and animal data, it is postulated that sex differences in the growth effects of androgens may be the result of changes in androgen signalling, particularly in the placenta where nutrient transport and growth mechanisms impact fetal growth.

3. Androgen signalling

Androgen signalling primarily occurs through the AR, a steroid receptor belonging to the nuclear receptor 3-ketosteroid group C (NR3C) subfamily. The *AR* gene has been localised to Xq11-12, contains eight exons and codes for a 919-amino acid protein (Fig. 2). Exon 1 of the *AR* gene encodes for the N-terminal domain (NTD): this domain contains an activation function 1 (AF1) region, which interacts with coregulatory proteins to enhance transcriptional regulation of AR-target genes. The second and third exons encode two distinct zinc-fingers (collectively referred to as the DNA-binding domain) required for interactions with androgen response elements (ARE) located within promoter regions of AR target genes. The remaining exons of the AR gene (4–8) encode a short flexible hinge region (which contains the nuclear localisation signal) and an alpha-helical sandwich, composed of 11 alpha-helices, which forms the AR C-terminal domain (CTD). This CTD is comprised of the ligand binding domain (LBD) and the AF2 co-regulator binding interface [42]. In its ligand-free state, the AR protein is localised in the cytoplasm where it is bound by a number of chaperone proteins including Hsp90 and immunophilins [43]. Upon interaction between androgen ligands and the LBD, a conformational change of the AR protein exposes the nuclear localisation signal in the hinge region, thereby allowing direct interactions with importin-α which facilitates subsequent nuclear translocation [44]. Within the nucleus, dimerization of ligand-bound AR units at promoter elements results in the transcriptional regulation of AR target genes, leading to either activated

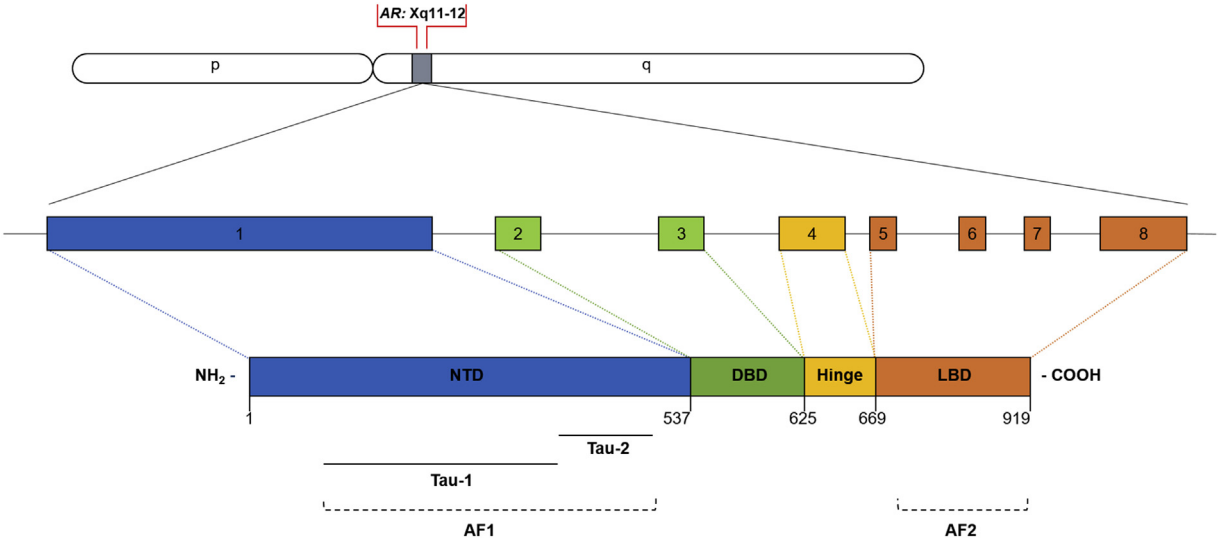

Fig. 2. – AR gene and protein schematic. The AR gene is localised to Xq11-12, contains eight exons, and codes for a 919-amino acid protein. The AR protein contains three major functional domains required for appropriate protein function. Exon 1 of the AR gene encodes for the N-terminal domain (NTD). The NTD contains two primary transactivation domains (transactivation uni-1 (Tau-1) and −2 (Tau-2)) within the activation function region 1 (AF1). Exon 2 and 3 of the AR gene encodes the DNA-binding domain (DBD) which interacts to the androgen response element (ARE) within the promoter region of target genes. This interaction is facilitated by two zinc fingers. Exon 4 of the AR gene encodes for a hinge region which contains the nuclear localisation signal that facilitates nuclear translocation. Exons 5–8 encode the ligand binding domain (LBD) which interacts with androgen ligands and also contains the AF2, which allows weak interactions with co-regulatory complexes.

or suppressed expression, depending on the co-regulatory proteins with which this AR-dimer is interacting [45].

There is a plethora of reported co-regulatory proteins that modulate transcriptional activity of AR-target genes. Prostate cancer research has demonstrated ligand dependent co-repressor (LCoR), forkhead box protein (FOX)A1, and FOXO1 modulate androgen-dependent and –independent AR activity [46,47]. It is unclear whether these protein-protein interactions are present in the human placenta and if a pregnancy complication affects their expression. Recent studies identified AR complexes containing histone lysine demethylases (KDMs) in ovine trophoblast cells [38]. This complex was shown to co-localise and bind to the ARE in the AR target genes VEGF and IGF-1. KDMs demethylate histone lysine residues increasing the accessibility of ARE on methylated AR target genes, which can enhance cell proliferation and growth [48]. Cleys et al. [38] observed that ewes treated with TP had significantly increased IGF-1 and VEGF mRNA and protein expression, when compared to controls. Furthermore, the same study identified KDM1A and KDM4D localisation in syncytiotrophoblast and villous stromal cells of first trimester human placentae. These findings suggest that AR-KDM complexes may modulate the transcriptional regulation of androgen-mediated target genes in human placental tissue cells, thereby contributing to growth stimulation by IGF-1 or VEGF-dependent mechanisms.

There is limited information about AR signalling in the human placenta and how interactions with co-regulatory proteins modulate AR function. Identification and characterisation of these interactions within the placenta may provide insight into the molecular mechanisms underpinning placental androgen signalling. Furthermore, how pregnancy comorbidities affect AR interactions with co-regulator proteins would assist in understanding sex-specific fetal-placental growth and developmental outcomes. While AR-coregulatory factors can modulate androgen-mediated signalling, another mechanism by which this pathway may be altered is through differential expression of AR transcript and protein variants.

4. AR variants

At present, there are at least 20 different AR transcript variants reported in the literature [49–55], which gives rise to numerous protein variants with molecular weights (MW) ranging from 45 to 120 kDa. Canonical genomic androgen signalling is mediated via the full-length AR protein (AR-FL). *In vitro* and *in vivo* studies have shown AR-FL necessitates growth in tumour and non-tumour tissue via regulation of downstream signalling pathways. Conversely, selective silencing of AR-FL has been reported to suppress tumour growth and proliferation. Hickey et al. [56] reported *in vitro* knockdown of AR-FL significantly reduced the proliferation of MDA-MB-453 cells. Similar findings have been reported in *in vitro* and *in vivo* models of other cancers including prostate [57] and bladder [58,59]. Testosterone stimulation of muscle-derived cells significantly increased IGF-1 mRNA expression and this response was abrogated by the AR-antagonist flutamide [60]. Very few studies have examined the expression of AR-FL in the human placenta and whether placental sex or pregnancy complications alter its expression. Horie et al. [61] reported the presence of AR in the human placental trophoblast, and Hsu et al. [62] reported AR localisation in the nucleus and cytoplasm of syncytiotrophoblasts and stromal cells. More recently preeclamptic pregnancies were reported to be associated with increased AR-FL protein and mRNA expression in the placenta [4], regardless of fetal sex.

Post-transcriptional modifications of AR can result in C− and N-terminally truncated variants. Alternative splicing of AR exons 1, 2, and cryptic exon (CE) 3 generate AR-V7. This C-terminally truncated, ligand-independent variant has been reported to regulate transcription of AR-FL target genes [56,63,64], as well as a unique subset of inflammatory genes, some of which include CXCL10, CCL5, IL8, and MMP13. AR-V7 overexpression in the breast cancer cell line MDA-MB-453 resulted in a gene expression profile that shared no overlap with gene expression profiles in the prostate cancer cell line LNCaP or MDA-MB-453 overexpressing AR-FL. These findings highlight the unique transcriptional regulatory role AR-V7 has and suggests this C-terminally truncated variant regulates specific mechanisms distinct from AR-FL. Expression of AR-V7 has primarily been reported in prostate and breast cancer tissue and cell lines, but it has also been found in non-tumour prostate tissue cells, suggesting a role in androgen signalling in normal tissue [63].

Similar to AR-V7, AR-V1 is produced through alternative splicing of

AR exons 1, 2, and CE1. AR-V1 lacks the C-terminal LBD and has been shown to inhibit AR-V7 by sequestering it in the cytoplasm. Co-overexpression of AR-V1 and AR-V7 suppressed the transcriptional activity of the AR-V7 specific gene, UBEC2C, demonstrating the inhibitory action of AR-V1 when expressed with AR-V7 [65]. In contrast, AR-FL dimerization with AR-V1 in LNCaP cells resulted in an increase in prostate specific antigen (PSA) mRNA expression, suggesting that the AR-V1/FL complex enhances the transactivational capacity of AR-FL in the presence or absence of androgens. Additional C-terminally truncated variants have also been reported (for a review, refer to Dehm & Tindall [54]), however the functions of these variants remain largely unknown.

AR-45 is a N-terminally truncated AR isoform, which is generated through alternative splicing of exon 1b, an intronically localised exon. AR-45 contains a unique seven amino acid sequence in place of the highly conserved NTD [66]. Studies assessing the function of AR-45 have demonstrated it suppresses cellular growth via a postulated AR-45/FL non-responsive heterodimer; however, the same study reported that in the androgen-independent cell line, PC-3, AR-45 co-expressed with TIF2 upregulated MMTV and PSA promoter activity in the presence of DHT or androstenedione [53]. Although AR-45 lacks the AF1, co-regulatory factors binding to the LBD-localised AF2 are able to enhance transcription of target genes [42,67,68]. Moreover, in the absence of AR-FL, AR-45 may function as a membrane-bound AR in neuronal SNpc and N27 cells mediating androgen-dependent non-genomic signalling [69]. To our knowledge, no study has reported multiple AR protein variants in the human placenta or in placentae of animal models. Given their capacity to modulate androgen-dependent and -independent signalling, assessing the expression and function of AR variants has been a focus within our group. Of particular interest is the differential expression of placental AR variants between the sexes and in the presence of pregnancy complications, and how these variants alter the placenta's response to androgens.

5. Placental androgen signalling in pregnancies complicated by asthma

Previous work from our team has identified that male fetuses grow normally and female fetuses reduce growth in the presence of maternal asthma [70]. We propose that this differential response is mediated by sex-specific alterations in placental function with male placentae inducing a state of glucocorticoid resistance to avoid the anti-proliferative effects of increased concentrations of endogenous glucocorticoids. Previous studies have indicated that increased fetal cortisol concentrations are positively correlated with testosterone levels in cord blood [3]. It is possible, therefore, that males continue to grow in the presence of maternal asthma or under stress conditions that result in a rise in cortisol because of androgen induced anabolic mechanisms in the placenta. We hypothesise that the growth difference between males and females is conferred by sex-specific differences in placental AR variant expression.

Preliminary data from our group has demonstrated sex-specific expression of placental AR variants which vary in the presence or absence of maternal asthma. Male placentae from asthmatic pregnancies had a significant reduction in the nuclear expression of three known AR isoforms including AR-FL, AR-V7 and AR-V1 but an increase in the nuclear expression of AR-45 [71]. There were no changes observed in female placentae which may suggest alternative pathways are prioritised in females that contribute to reduced growth in the presence of maternal asthma. Interestingly, increased AR-45 expression in male placentae from asthmatic pregnancies did not appear to inhibit mRNA expression of androgen-mediated target genes including *IGF-1*, *IGF-1R*, *IGFBP-3*, *IGFBP-5* or *VEGF*; in fact, the data demonstrated that the presence of maternal asthma increased expression of these growth targets, and correlation data – as well as preliminary *in vitro* studies – would suggest AR-45 may be involved in *IGF-1R* and *IGFBP-5* transcription (unpublished data). Our preliminary findings justify further investigations to explore the mechanistic function of placental AR variants, which may provide evidence supporting a crucial role of AR protein variants in placental pathophysiology.

6. Conclusion

It is evident that the placental androgen action is mediated by a complex network of pathways that remain to be fully understood. The involvement of androgens in male and female intrauterine growth and development has been documented, and it is clear that pregnancy co-morbidities can perturb placental androgen signalling and give rise to fetal morbidities (or, in extreme cases, mortality) and may contribute to the development of diseases later in life. Our group's findings suggest that canonical androgen signalling is not the established mechanism of androgen action in the human placenta, which necessitates further investigations of the role of AR variants in the human placental function. Understanding the complexity of placental androgen signalling and its role in sex-specific growth and developmental outcomes, especially in circumstances where a specific pathophysiology of pregnancy exists, is critical.

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Appendix A. Supplementary data

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