Placenta 80 (2019) 18–26

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Human placental androgen receptor variants: Potential regulators of male fetal growth

Ashley S. Meakin[a], Zarqa Saif[a], Astrud R. Tuck[b], Vicki L. Clifton[a,*]

[a] Pregnancy and Development, Mater Medical Research Institute-University of Queensland, Brisbane, Australia
[b] Robinson Research Institute, University of Adelaide, Adelaide, Australia

ARTICLE INFO

Keywords:
Androgen receptor
Fetal growth
Asthma
Placenta
Pregnancy
Sex

ABSTRACT

Introduction: Numerous studies show that males have increased intrauterine growth compared to females, and that pregnancy complications may further these growth differences, but the regulatory mechanisms underlying these differences remain unknown. We propose that these growth outcomes may be due to sex-specific differences in androgen sensitivity – giving rise to altered growth signalling pathways – mediated by the differential expression of placental androgen receptor (AR) variants.

Methods: Placental protein and mRNA were used to identify AR protein variant levels and AR-downstream target gene expression, and were then analysed against neonatal measurements. Dihydrotestosterone (DHT)-induced AR protein variant expression and downstream growth factors were examined *in vitro*.

Results: Four known AR variants (AR-FL, AR-V1, AR-V7, and AR-45), and three unknown proteins (120, 90 and 55 kDa) immunoreactive to the anti-AR antibody were identified in human placentae. Male placentae from controlled asthmatic pregnancies had increased AR-45 and decreased AR-V1 and AR-V7 nuclear expression. Increased nuclear AR-45 expression was associated with increased insulin-like growth factor 1 (*IGF-1*), IGF-1 receptor (*IGF-1R*), and IGF-binding protein 5 (*IGFBP-5*) mRNA expression and normal male growth. AR-45 mRNA and protein did not change in the presence of uncontrolled maternal asthma and associated with an increase in small for gestational (SGA) male fetuses. *In vitro* DHT stimulation increased AR-45 protein and *IGF-1R* and *IGFBP*-5 mRNA expression.

Conclusions: Collectively, our data shows altered AR protein expression and downstream signalling targets may contribute to sex-specific fetal growth outcomes in response to an adverse environment, and that AR-45 appears central in mediating these changes.

1. Introduction

Current epidemiological data shows sex-specific fetal growth differences with males, on average, having increased birthweight (BW) and birthweight centile (BWC) compared to females in normal pregnancies [1]. The mechanisms by which these growth differences are regulated remain to be fully defined, however it is postulated that alterations to placental androgen signalling may be integral for increased male growth relative to female growth [2].

Androgens act via the androgen receptor (AR), a steroid receptor belonging to the nuclear receptor 3-ketosteroid group C (NR3C) subfamily. *AR* contains eight exons and codes for a 919-amino acid protein that can regulate transcription of genes associated with several functions, some of which include cell growth, apoptosis, and signal transduction [3,4]. During pregnancy, placental and myometrial androgen synthesis may stimulate AR regulatory pathways necessary for cellular development and differentiation [5]. Androgen signalling disruption via complete AR-knockout perturbs intrauterine growth and development in mice, with the greatest adverse outcomes observed in male offspring [6]. Likewise, an AR mutation in 46,XY neonates is associated with reduced birthweight and genital abnormalities [7].

Although *AR* mRNA expression is ubiquitous, receptor protein expression is tissue-specific with the functional full-length AR (AR-FL) variant (110 kDa) primarily expressed in female and male reproductive tissue cells [5,6]. Immunohistochemistry identified AR in syncytiotrophoblast and vascular endothelial cells of the human placenta [8], and Western blot identified AR-FL in placentae from normal and preeclamptic pregnancies [9], however no studies have reported expression of other AR variants within the human placenta.

At present, there are at least 20 different AR transcript variants

---

[*] Corresponding author. Mater Medical Research Institute, Level 4, Translational Research Institute, 37 Kent St, Woolloongabba, Qld, 4101, Brisbane, Australia.
E-mail address: vicki.clifton@mater.uq.edu.au (V.L. Clifton).

https://doi.org/10.1016/j.placenta.2019.03.012
Received 29 January 2019; Received in revised form 12 March 2019; Accepted 25 March 2019

reported [10–14]. A number of these identified AR protein variants have unknown function, however some C- [15] and N-terminally [13] truncated variants have been identified in reproductive organs and are shown to modulate androgen cellular responses. Although these variants have been identified and characterised in adult tissues, it is unknown if they are expressed in the placenta and whether they are altered by a pregnancy complication such as maternal asthma.

Asthma is a common co-morbidity to affect pregnant individuals, with 12% of Australian pregnancies [16], and 3–12% worldwide [17], affected by this disease. One-third of asthmatic, pregnant individuals experiencing an increase in the disease's severity and 50% are reported as having uncontrolled symptoms [18]. Loss of asthma control and an increase in the rate of asthma exacerbations has been shown to contribute to an increase in fetal and maternal cortisol concentrations, maternal systemic inflammation, increased oxidative stress, reduced maternal oxygen levels, and maternal alkalosis [19]. These maternal risks can result in poor outcomes for the developing fetus including low birthweight (< 2500 g) (LBW), small for gestational age (< 10th BWC) (SGA), and preterm delivery [20].

Studies from our group investigating the effect of maternal asthma on fetal development demonstrate that the risk of an adverse outcome is sex-dependent [2]. In the presence of maternal asthma females reduce growth and alter expression of placental genes associated with immunity and inflammation through a greater sensitivity to glucocorticoids, providing survival advantages in the event of subsequent asthma exacerbations. In contrast, males continue to grow with minimal placental function changes, which is postulated to contribute to a greater risk of adverse antenatal and perinatal events [19]. The mechanisms that contribute to these sex-specific growth differences in the presence of maternal asthma remain unclear, however we hypothesise that alterations to placental androgen signalling, mediated via altered placental AR variant expression, may confer these differences. Therefore, in the current study we have examined the expression of placental AR variants and determined whether they are altered by maternal and neonatal variables, and whether these AR variants are associated with changes in placental and neonatal measures.

2. Materials and methods

2.1. Maternal cohort recruitment, asthma assessment, and placenta collection

Asthmatic and non-asthmatic individuals were recruited at the beginning of pregnancy (non-asthmatic n = 39, asthmatic n = 61). Participant data was collected throughout pregnancy as previously described [18]. Placentae were collected and processed at the time of delivery as previously described [21]. Ethics for this project was approved by the Lyell McEwin Hospital and Queen Elizabeth Hospital Ethics Committee, and the University of Adelaide.

2.2. Cell culture

Human prostate cancer LNCaP cells [22] and human cytotrophoblast BeWo cells [23] were maintained in RPMI-1640 or DMEM-F12, respectively.

2.2.1. In vitro AR knockdown

BeWo cells were cultured to 70% confluency in a 6-well plate. On the day of transfection, culture media was replaced with 1.5 ml of OPTI-MEM with 5% FBS six hours prior to transfection with an AR-specific ([Assay ID: 146896], Thermo Fisher Scientific, USA) or scrambled siRNA using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, USA). Cells were harvested 48 h' post-transfection and pellets were stored at −30 °C until analysed.

2.2.2. In vitro androgen stimulation

BeWo cells were cultured to 70% confluency in individual wells on a 6-well plate. Media was then changed to DMEM-F12 supplemented with 5% charcoal stripped serum (CSS). Cells were cultured for 48 h, and then cultured in CSS DMEM-F12 supplemented with 0, 0.01, 0.1, 1 or 10 nM dihydrotestosterone (DHT) (Sigma-Aldrich, USA) or vehicle control. Cells were cultured for an additional 24 h and then pellets were collected as aforementioned.

2.3. Western blot

2.3.1. Cellular fraction preparation

Placental tissue was analysed from control (n = 23) and asthmatic (n = 41) pregnancies. Cellular (BeWo and LNCaP) total lysates and cellular and placental cytoplasmic and nuclear fractions were prepared as previously described [21].

Lysates (50 μg) were electrophoresed on 3–8% Tris-acetate precast gels (Invitrogen, Life technologies, Carlsbad, California, USA) as previously described [21]. Antibodies used in this study include: anti-AR sc-816 (Santa Cruz, USA); anti-AR ab74272 (Abcam, UK); anti-AR C19 (Santa Cruz, USA); anti-β-actin ab191866 (Abcam, UK); anti-Lamin A/C sc-2020 (Santa Cruz, USA); Goat anti-Rabbit IgG H&L (HRP) ab97051 (Abcam, UK); and Mouse anti-Rabbit IgG VHH Single Domain (HRP) ab191866 (Abcam, UK). Densitometric analysis was conducted using BIO-1D gel analysis software (Vilber Lourmat, France) to determine expression of AR protein bands relative to β-actin.

2.3.2. Peptide competition of AR antibody

Peptide competition was performed using the anti-AR sc-816 antibody (Santa Cruz, USA). sc-816 (100 μg/0.1 ml) was incubated with 20× (2 mg) concentration of the control peptide (Santa Cruz, USA) for 2 h with rotation at room temperature. Complexes were then centrifuged at 13000 rpm for 5 min and incubated on the Western blots of placental samples. Data was compared to sc-816 alone, incubated in parallel.

2.4. Real-time quantitative PCR analysis

Placental tissue RNA extraction from control (n = 39) and asthmatic (n = 61) pregnancies, and cell RNA extraction from BeWo cells, was completed as previously described [24]. cDNA was synthesised from 1 μg RNA using the SuperScript IV First-Strand Synthesis Kit (Thermo Fisher Scientific, USA). RT-qPCR TaqMan Assay® (Applied Biosystems™) reactions were run in duplicates as per the manufacturer's protocol. A summary of TaqMan Assay® (Applied Biosystems™) sequence specific primers and the respective assay IDs can be found in Supplementary Table 1. Target gene expression was normalised against β-actin using C_t values and relative expression was calculated using formula $2^{-(\Delta Ct)}$.

2.5. Data analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS v 22). Where possible, data was analysed by asthmatic subgroups including severity (the presence or absence of an exacerbation) or control (the presence or absence of inhaled corticosteroid (ICS) use). Protein and mRNA data was not normally distributed so non-parametric tests including KW-ANOVA, Mann-Whitney *U* Test, Bonferroni post-hoc analyses, and Spearman's correlations were performed. For normally distributed data, student T-tests and one-way ANOVA with Bonferroni post-hoc analysis was performed. All data were presented as medians with interquartile ranges, unless stated otherwise. The alpha level was set at 0.05.

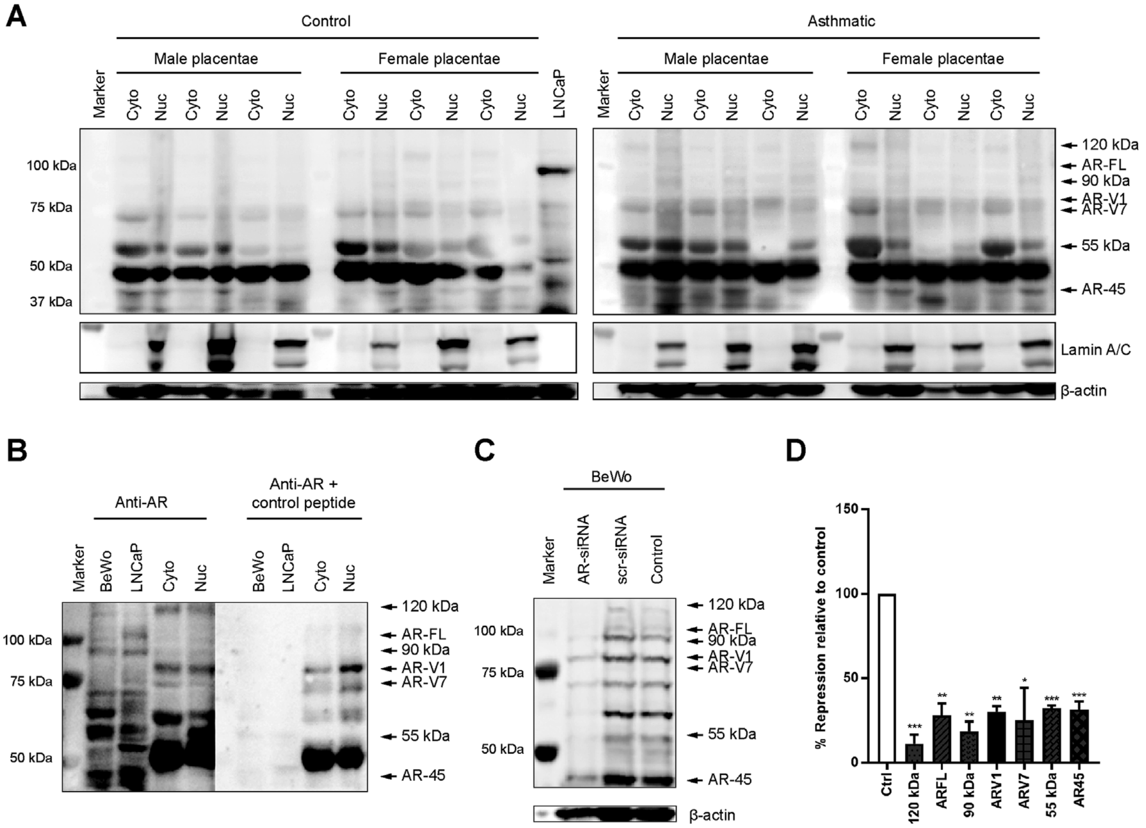

Fig. 1. Expression and validation of multiple androgen receptor (AR) isoforms within the human placenta. A) Nuclear and cytoplasmic protein extracts of male and female placentae from control, non-asthmatic pregnancies and pregnancies complicated by asthma. Seven proteins were detected using the sc-816 anti-AR antibody ranging from 45 kDa to 120 kDa. Blots were further probed with Lamin A/C as a nuclear marker and β-actin as a loading control. **B)** Peptide pre-absorption of sc-816 antibody with 20× (2 mg) control peptide. **C)** BeWo cells were transfected with either a control siRNA or AR specific siRNA and AR isoforms were measured using Western blot using AR specific antibody and B-actin as housekeeping control. **D)** Normalised densitometric values from three experiments from AR specific siRNA were plotted as a percentage of repression relative to the control siRNA values. Bars are mean percentage repression relative to control and SEM. Statistical significance: * = $p \leq 0.05$, ** = $p \leq 0.01$, ** = $p \leq 0.001$.

3. Results

3.1. Maternal and neonatal characteristics

Baseline and predicted (%) forced expiratory volume at one second (FEV1) was significantly lower in controlled, asthmatic individuals carrying a female fetus ($p < 0.05$) (Supplementary Table 2). Maternal age was significantly lower in asthmatic individuals carrying a female fetus who experienced an exacerbation ($p < 0.05$) (Supplementary Table 2). No other maternal characteristics varied significantly. No neonatal characteristics varied significantly between the groups, however there was an observed rise in the percentage of SGA neonates if maternal asthma was uncontrolled (Supplementary Table 3).

3.2. AR variants expressed in the human placenta

Seven protein bands immunoreactive to anti-AR antibodies were identified in cytoplasmic ($n = 64$) and nuclear ($n = 64$) fractions of whole placentae at molecular weights (MW) of 120, 110, 90, 76, 75, 55, 45 kDa (Fig. 1A). LNCaP total lysates were used as a positive control as previous studies have demonstrated expression of multiple AR variants in this cell line: expression of the seven immunoreactive protein bands was also detected in this control (Fig. 1A). Four of the seven identified bands represent previously known and characterised AR variants (AR-FL (110 kDa), AR-V1 (76 kDa), AR-V7 (75 kDa) and AR-45 (45 kDa)), with AR-V1, AR-V7, and AR-45 expression significantly higher compared to AR-FL, regardless of maternal asthma or fetal sex (data not shown). Following peptide pre-absorption of the AR antibody sc-816, all protein bands, excluding the 50 kDa protein band, were either reduced or completely blocked in BeWo and LNCaP cell lines, as well as cytoplasmic and nuclear human placental fractions (Fig. 1B). BeWo cells were transfected with AR siRNA treatment as another means of assessing whether all immunoreactive AR antibody proteins were potential AR variants. There was a significant reduction in all seven protein bands that were immunoreactive to the anti-AR antibodies (Fig. 1Cand D).

3.3. Effect of maternal asthma and fetal sex on AR variant expression

The 55 kDa AR protein was significantly higher in female control placentae compared to male ($p = 0.05$) but this change was not observed in female placentae of pregnancies complicated by asthma (data not shown). No other AR variants were altered in female placentae in the presence or absence of maternal asthma. There were no significant changes in placental AR protein expression between asthma treatment groups; however, cytoplasmic AR-45 expression was significantly reduced in asthmatic pregnancies that did not experience an exacerbation relative to non-asthmatic controls ($p = 0.05$) (data not shown).

In male placentae in the presence of maternal asthma, there was a significant decrease in nuclear localised AR-FL, 90 kDa, AR-V1, and AR-V7 relative to controls (Fig. 2A). Further, nuclear localised AR-V1 and AR-V7 were significantly reduced in placentae when severity and control was considered, relative to non-asthmatic controls (Fig. 2B).

When analysing the relative ratio of individual cytoplasmic and nuclear AR proteins, only nuclear localised AR-45 significantly increased in male placentae from pregnancies complicated by asthma

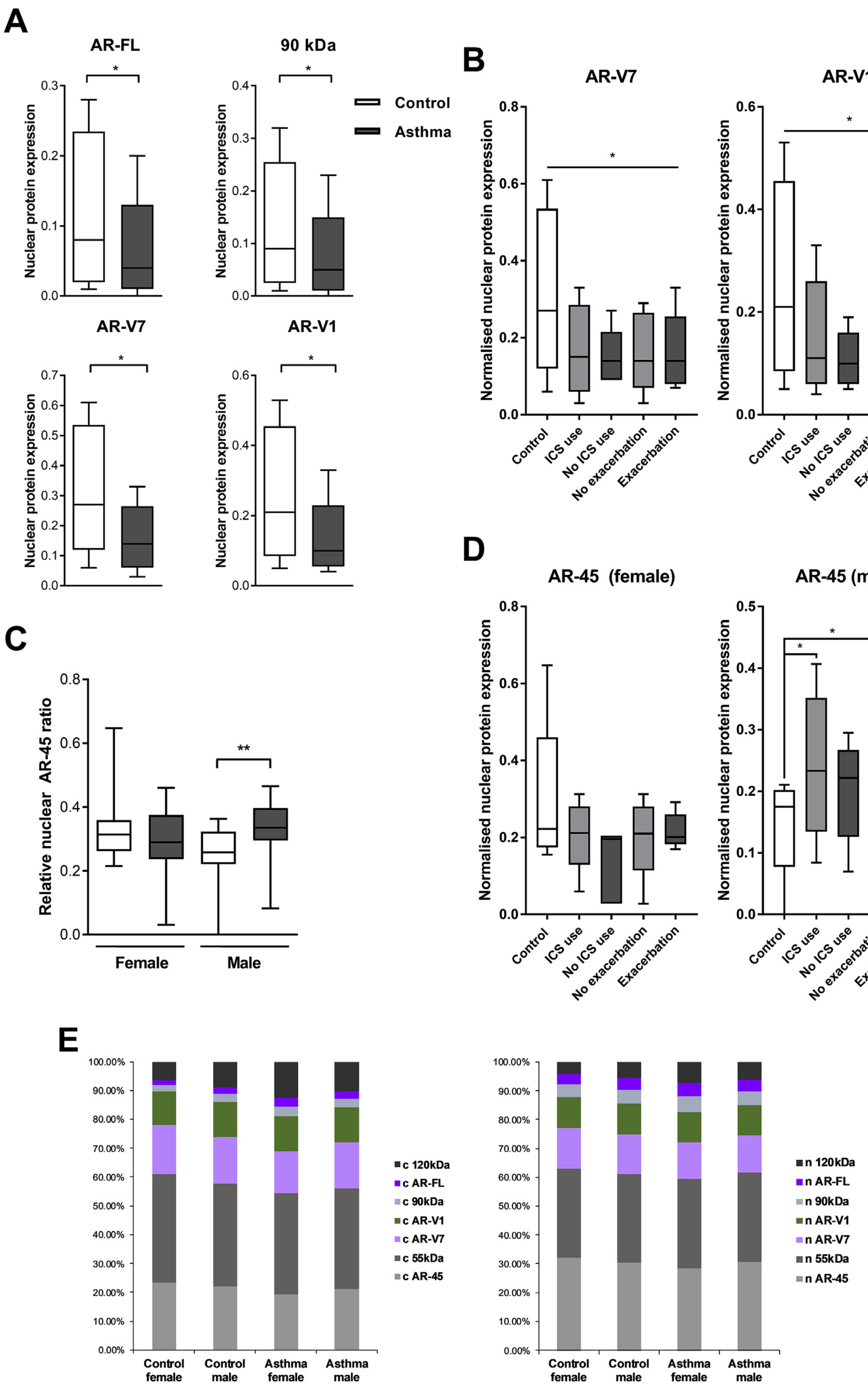

Fig. 2. Placental AR isoform expression is altered by maternal asthma status in male placentae. A) Previously identified and characterised AR proteins (AR-FL, AR-V1, AR-V7) and the unknown 90 kDa protein had significantly decreased expression in male placentae from asthmatic pregnancies (closed bars) compared to control (open bars). Statistical analysis: Mann-Whitney *U* Test. Statistical significance: * = $p \leq 0.05$. **B)** Nuclear expression of AR-45 in males from asthmatic pregnancies, split by ICS use and exacerbation. **C)** Relative expression of nuclear AR-45 protein expression in males from asthmatic (closed bar) and control (open bar) pregnancies. **D)** Relative expression of nuclear AR-45 protein expression in males and females from asthmatic pregnancies, split by ICS use and exacerbation. **E)** Mean % expression of all individuals (n = 64) separated by fetal sex and maternal asthma status in the cytoplasm and nucleus. Statistical analysis: Mann-Whitney *U* Test. Statistical significance: ** = $p \leq 0.01$.

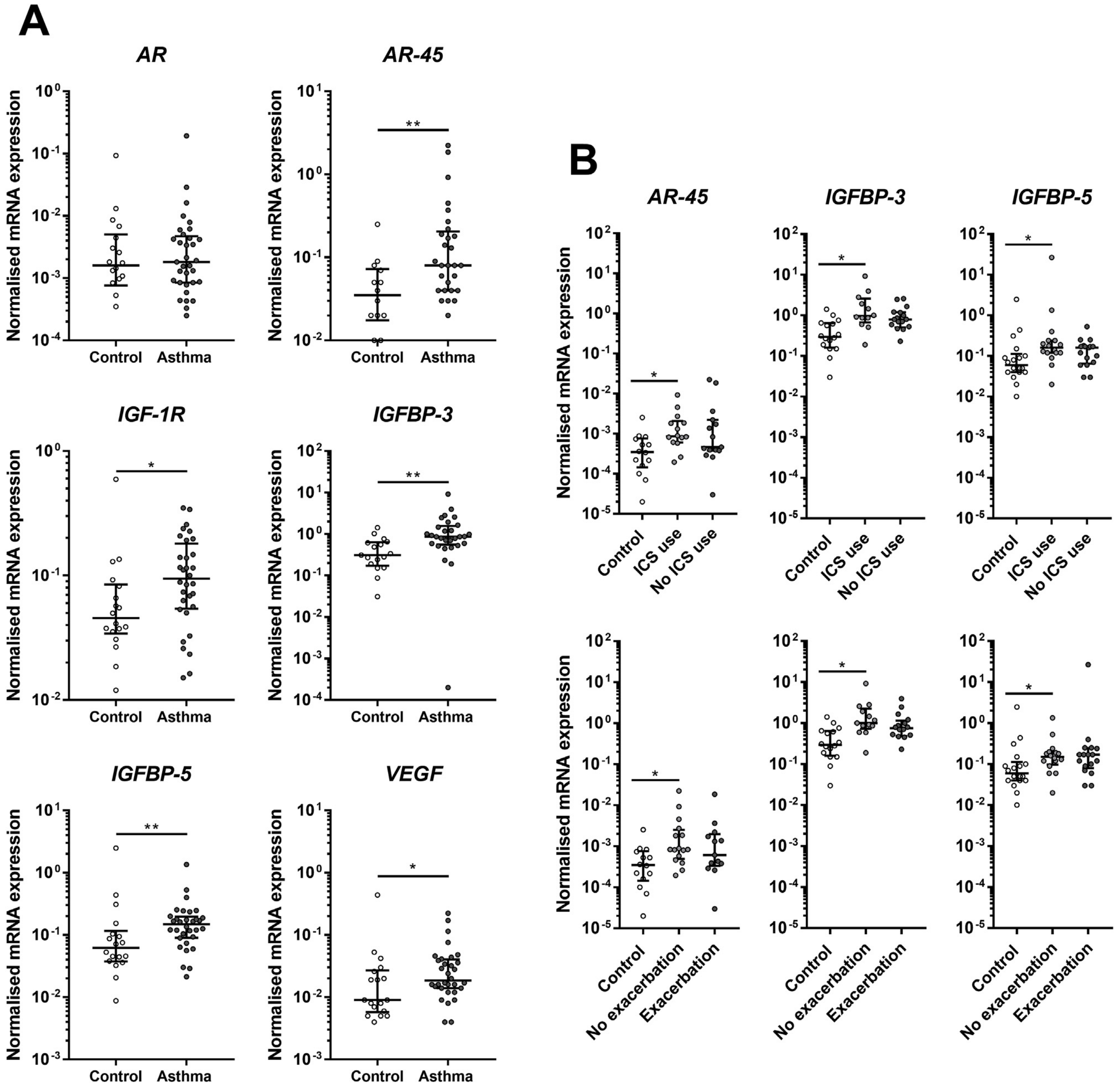

Fig. 3. Expression of *AR* mRNA and AR-regulated downstream target genes. A) Expression of *AR*, *AR-45*, *IGF-1R, IGFBP-3*, *IGFBP-5*, and *VEGF* mRNA in male and placentae from control and asthmatic pregnancies. **B)** Expression of *AR-45*, *IGFBP-3*, and *IGFBP*-5 mRNA in male placentae from asthmatic pregnancies, split by ICS use and exacerbation. mRNA data was normalised against B-actin mRNA. Statistical analyses: Mann-Whitney *U* Test, Kruskal-Wallis pairwise comparison. Statistical significance: * = $p \leq 0.05$, ** = $p \leq 0.01$.

(Fig. 2C). The same results were reported when asthma severity or treatment was considered (Fig. 2D). When the same analysis was conducted on female placentae, there were no significant changes in known AR protein expression. When examining the expression of all AR proteins as a percentage of the total placental AR expression, the 55 kDa protein predominated in the cytoplasm of male and female placentae, whereas AR-45 and the 55 kDa protein accounted for more than 50% of the total AR protein detected within the nucleus of both sexes (Fig. 2E).

3.4. AR-regulated downstream signalling pathways expression

AR-45, insulin-like growth factor (IGF) 1 receptor (*IGF-1R*), IGF binding protein (IGFBP) 3 (*IGFBP-3*), *IGFBP-5*, and vascular endothelial growth factor (*VEGF*) mRNA expression was significantly increased in male placentae from asthmatic pregnancies compared to controls (Fig. 3A). *AR-45*, *IGFBP-3*, and *IGFBP*-5 mRNA expression was significantly increased when asthma was controlled or uncomplicated by an exacerbation (Fig. 3B). Maternal asthma status, its control, or severity did not affect gene expression in female placentae (Data not shown).

3.5. Relationships between AR, downstream signalling pathways and growth

Correlations between placental total *AR* and *AR*-45 mRNA and downstream signalling targets including *IGF-1*, *IGF-1R*, *IGFBP-5*, and *VEGF* mRNA were observed in females and males from asthmatic and non-asthmatic pregnancies (Table 1). Nuclear AR-FL protein negatively correlated with *IGF-1R* and *IGFBP*-1 mRNA in female placentae from pregnancies complicated by asthma only (Table 1). There were no correlations of significance between nuclear AR-FL and target gene

Table 1

Total AR and AR-45 mRNA, and AR-FL and AR-45 nuclear protein correlates with *IGF-1*, *IGF-1R*, *IGFBP-1*, *IGFBP-3*, *IGFBP-5*, and *VEGF* mRNA expression. Normalised mRNA of total AR and AR-45 was correlated with target genes *IGF-1, IGF-1R, IGFBP-1, IGFBP-3, IGFBP-5,* and *VEGF* mRNA to assess differences in the relationship between total AR and AR-45. Similarly, nuclear localised AR-FL and AR-45 protein was correlated with the aforementioned target genes to assess the relationship they have with target gene expression. Statistical analysis: Spearman's correlation. Statistical significance: * = p ≤ 0.05, ** = p ≤ 0.01.

	Total *AR* mRNA								*AR*-45 mRNA							
	Control				Asthma				Control				Asthma			
	Female		Male		Female		Male		Female		Male		Female		Male	
	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
IGF1	−0.371	n.s	0.444	n.s	0.287	n.s	**0.709**	**	0.215	n.s	0.106	n.s	**0.523**	**	**0.658**	**
IGF1R	0.241	n.s	0.331	n.s	**0.473**	*	**0.530**	**	**0.712**	**	0.521	n.s	**0.770**	**	**0.672**	**
IGFBP1	0.153	n.s	0.364	n.s	0.407	n.s	**0.453**	*	0.003	n.s	−0.224	n.s	0.130	n.s	0.359	n.s
IGFBP3	0.182	n.s	0.300	n.s	0.196	n.s	**0.486**	*	**0.773**	**	0.214	n.s	0.255	n.s	0.326	n.s
IGFBP5	−0.020	n.s	0.176	n.s	0.217	n.s	**0.425**	*	0.144	n.s	0.257	n.s	**0.498**	**	**0.602**	**
VEGF	−0.018	n.s	0.427	n.s	**0.585**	**	**0.544**	**	0.353	n.s	0.055	n.s	**0.504**	**	**0.541**	**
	Nuclear AR-FL protein								Nuclear AR-45 protein							
	Control				Asthma				Control				Asthma			
	Female		Male		Female		Male		Female		Male		Female		Male	
	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p	r^2	p
IGF1	0.129	n.s	0.240	n.s	−0.459	n.s	−0.132	n.s	0.358	n.s	0.225	n.s	0.028	n.s	**0.458**	*
IGF1R	0.073	n.s	−0.394	n.s	**−0.704**	**	−0.440	n.s	−0.145	n.s	0.261	n.s	0.104	n.s	**0.561**	*
IGFBP1	0.005	n.s	0.152	n.s	**−0.713**	**	−0.332	n.s	−0.200	n.s	−0.067	n.s	0.100	n.s	0.343	n.s
IGFBP3	0.565	n.s	0.217	n.s	0.011	n.s	−0.077	n.s	0.200	n.s	0.000	n.s	−0.240	n.s	0.247	n.s
IGFBP5	0.396	n.s	0.055	n.s	−0.104	n.s	0.139	n.s	0.300	n.s	0.442	n.s	−0.411	n.s	**0.489**	*
VEGF	0.329	n.s	−0.209	n.s	−0.196	n.s	−0.140	n.s	0.073	n.s	0.092	n.s	−0.206	n.s	0.327	n.s

mRNA expression in the male population. However, nuclear localised AR-45 protein positively correlated with *IGF-1*, *IGF-1R*, and *IGFBP*-5 mRNA in male placentae from pregnancies complicated by asthma but these relationships were not observed in control male populations (Table 1).

Correlations between AR proteins and additional markers of growth were analysed. Male BWC from pregnancies complicated by asthma positively correlated with cytoplasmic AR-45 ($R^2 = 0.535$, $p = 0.010$), and negatively correlated with nuclear AR-FL ($R^2 = -0.529$, $p = 0.011$) (Fig. 4A). Negative correlations were observed between AR-V7 and head circumference (cm) ($R^2 = -0.484$, $p = 0.022$) and AR-V7 and birth length (cm) ($R^2 = 0.447$, $p = 0.037$) in males, whereas positive correlations between AR-V7 and head circumference ($R^2 = 0.674$, $p = 0.003$) and AR-V7 and birth length ($R^2 = 0.605$, $p = 0.010$) were observed in females (Fig. 4B).

3.6. *In vitro trophoblast androgen signalling*

To further investigate the role of androgen-dependent signalling on the placental AR expression and downstream signalling pathways, BeWo cells were stimulated with increasing concentrations of DHT (Fig. 5A). The AR variants detectable in BeWo cells included AR-FL, AR-V1, AR-V7, AR-45, as well as the 90 and 55 kDa protein bands. AR-45 protein expression was significantly increased at 0.1 nM DHT and significantly decreased at 10 nM DHT, when compared with the unstimulated control (Fig. 5B). Expression of AR-45 was significantly increased at 0.1 nM, when compared with the other DHT concentrations (Fig. 5C). Protein expression of other AR variants did not change in the presence of DHT (Data not shown). The AR regulated genes *IGF-1R* and *IGFBP-5* were significantly increased in the presence of 0.1 nM DHT when compared to unstimulated cells (Fig. 5D). AR-45 protein was primarily localised to the nucleus of BeWo cells in the presence of DHT (Fig. 5E).

4. Discussion

To our knowledge, this is the first study to identify multiple AR protein variants in the human placenta which vary by fetal sex, cellular localisation, and in the presence or absence of the complication of asthma. Several known AR variants were present in the placenta including AR-FL, AR-V7, AR-V1, and AR-45. Additionally, expression of protein bands at 120, 90 and 55 kDa were identified, however the function of these AR variants remain undefined although their expression has been previously reported in other tissue and cell lines [14,25,26]. In the presence of maternal asthma, male placentae had decreased expression of AR-FL, AR-V1, and AR-V7, and the 90 kDa protein. AR-45 was the only AR variant to have increased expression in male placentae in the presence of asthma. This increase was also observed in asthmatic pregnancies controlled with ICS use or uncomplicated by an exacerbation. In conjunction with this increased AR-45 expression, there was a rise in androgen regulated targets including *IGF-1R*, *IGFBP-3*, *IGFBP-5* and *VEGF* [4,27–29]. These data suggest a potential role for AR-45 and certain downstream targets in regulating male intrauterine growth in controlled, asthmatic pregnancies.

AR-45 is an androgen-responsive, N-terminally truncated variant that results from alternative initiation of translation at exon 1B, an exon located within intron 1 of AR, and is thought to antagonise AR-FL [13,30]. Studies have also shown, however, that this variant can directly transactivate AR target genes under certain cellular conditions: AR-45 was reported to stimulate mouse mammary tumour virus (*MMTV*) and prostate specific antigen (*PSA*) promoter activity in the presence of increased androgen derivatives and cofactors in the AR-null prostate cancer cell line PC-3 suggesting the function of this variant may be situational [13]. In the placenta, it appears to function sex-specifically and may be involved in regulating continued male growth, particularly in the presence of maternal asthma that is managed by ICS use and uncomplicated by an exacerbation. Evidently, further functional characterisation studies of this AR variant within the placenta are required.

In the presence of maternal asthma, the only other detected AR protein variants that had altered expression were the C-terminally truncated variants AR-V1 and AR-V7. Their expression negatively correlated with male neonatal growth markers including head circumference and birth length. Previous studies have characterised these

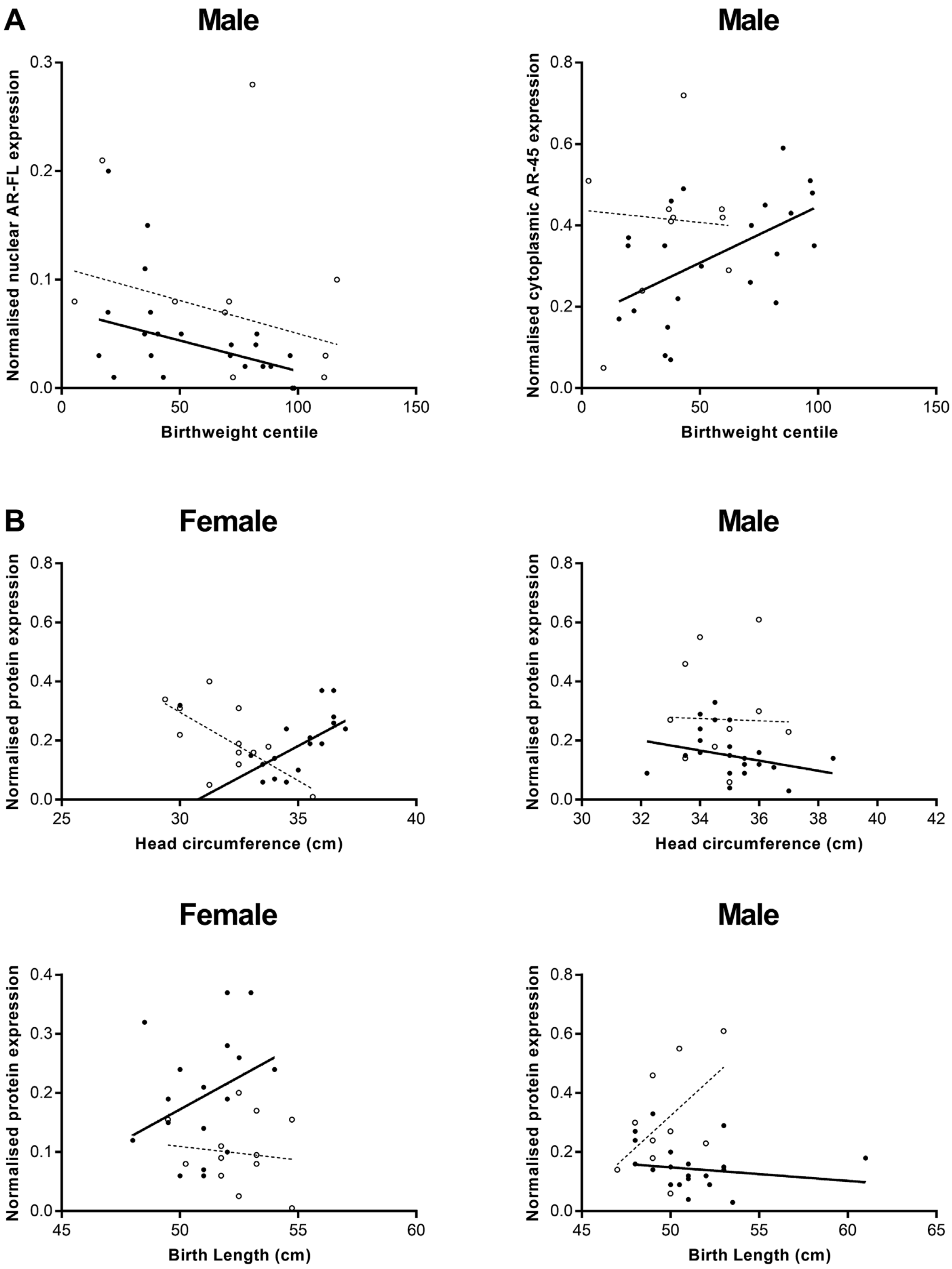

Fig. 4. Fetal markers of growth associate with fetal-placental variables. Open data points and dashed trend lines represent the control population, whereas closed data points and unbroken trend lines represent the asthmatic population. **A)** Nuclear AR-FL negatively correlates with birthweight centile (BWC) in male placentae from asthmatic pregnancies, and nuclear AR-FL negatively trends with BWC in male placentae from control pregnancies, whereas cytoplasmic AR-45 positively correlates with BWC in male placentae from asthmatic pregnancies only. **B)** Female and male placental nuclear AR-V7 correlate with fetal head circumference (cm) and birth length. All protein data was normalised against β-actin. Statistical analysis: Spearman's correlation.

variants in prostate and breast cancer and demonstrated that they regulate transcription of AR-FL target genes, as well as a unique subset of inflammatory genes, some of which include inhibition of C-X-C motif chemokine 10 (*CXCL10*), chemokine (C-C motif) ligand 5 (*CCL5*), interleukin 8 (*IL8*), and matrix mettalopeptidase 13 (*MMP13*) [31]. In the context of our study, reduced AR-V7 expression in male placentae may be a mechanism implemented to regulate placental inflammation and ensure appropriate transactivation of androgen-dependent target genes involved in growth pathways. Unlike AR-V7, the function of AR-V1 remains in question: it is thought to antagonise AR-V7 while enhancing

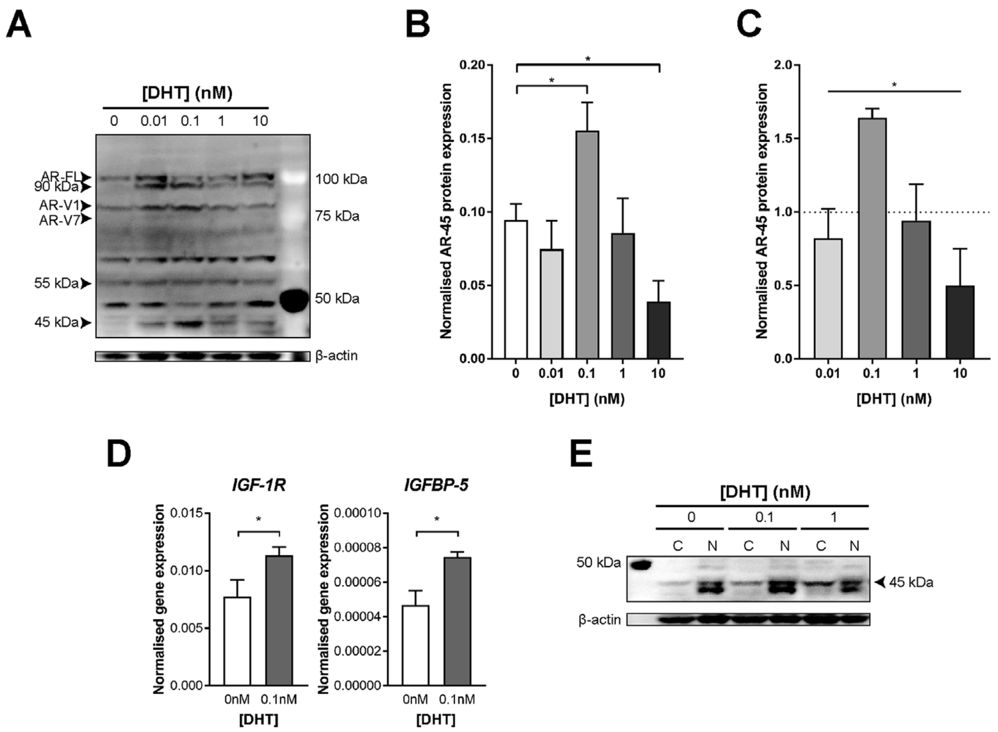

Fig. 5. DHT stimulation increases AR-45 protein expression and downstream target genes in an *in vitro* trophoblast model. A) Representative Western blot figures of BeWo cells stimulated with increasing concentrations of DHT. Detected protein bands were normalised against β-actin. **B)** Normalised protein expression of AR-45 in response to changing concentrations of DHT. **C)** Relative protein expression of AR-45 from DHT stimulated BeWo cells. Data is represented as a relative percentage of expression of unstimulated control cells. **D)** Normalised expression of AR-associated target genes in unstimulated and 0.1 nM DHT stimulated BeWo cells. **E)** Cytoplasmic and nuclear fraction of BeWo cells stimulated with increasing concentrations of DHT. Data is representative of four individual experiments. Data is displayed as mean with SEM. Statistical analyses: One-way ANOVA and Student's T-test. Statistical significance: * = p ≤ 0.05.

AR-FL transcriptional regulation [32], although further studies are required to ascertain the function of these C-terminally truncated AR variants within the human placenta, and whether they are involved in regulating sex-specific intrauterine growth.

The trophoblast cell response to DHT exposure *in vitro* was associated with increased AR-45 expression and nuclear localisation, and a rise in some growth factor pathways including *IGF-1R* and *IGFBP-5*. Interestingly, this response was concentration dependent, with the greatest induction of AR-45 expression observed at 0.1 nM DHT, a concentration that falls in the range of physiological levels throughout gestation [5]. It is important to note that the cell line used throughout this study, BeWo, is a male trophoblast cell line [23], making it an appropriate *in vitro* model for understanding placental mechanisms that contribute to a continued male growth phenotype. However, future studies would benefit from utilising primary isolated trophoblasts to ascertain sex-specific androgen-mediated mechanisms that contribute to growth outcome differences. This *in vitro* work, in conjunction with the observed correlations between nuclear AR-45 and target genes in male placentae from asthmatic pregnancies, supports the concept that placental AR-45 may be physiologically relevant in contributing to growth during pregnancy, potentially via the transactivation of genes involved in IGF-signalling.

IGF-1R is a known androgen-dependent target that regulates growth [29,33]. IGF-1R binding of IGF-1 activates pathways involved in cellular growth and proliferation, including the mammalian target of rapamycin (mTOR) [34–36]. Placental mTOR is involved in nutrient transfer to the developing fetus. Its overexpression is associated with large for gestational age (LGA) placentae and fetuses, whereas reduced expression has been associated with perturbed intrauterine growth [37,38]. Findings from the current study suggest human placental IGF-1R is sex-specifically regulated and responsive to androgens and may be central for male intrauterine growth in the presence of maternal asthma, regardless of severity or control, however further investigations are needed.

Unlike IGF-1R, IGFBP-5's involvement in growth is yet to be fully defined. IGFBPs function to sequester and prolong the half-life of IGF-1 [39]; however, studies would suggest certain IGFBPs may directly regulate growth via unique IGF-1-indepdendent pathways. *In vivo* studies have demonstrated IGFBP-5 stimulates cell-specific growth via IGF-1-independent mechanisms [40,41], although it is unclear whether IGFBP-5 functions in this manner within the placenta, and whether sex or the presence of maternal asthma modulates its function.

While this prospective pregnancy cohort study is limited to associations between AR protein variant expression, downstream target genes, and clinical data, our findings highlight that certain variants may be involved in the sex-specific regulation of growth pathways in the human placenta and gives precedence for further functional characterisation studies. In conjunction with the associations observed in the placenta cohort, we have demonstrated in preliminary *in vitro* characterisation studies that AR-45 may have a critical role in regulating the transcription of downstream growth targets, however further characterisation of these signalling pathways is required.

From our findings we propose that there is a placental adaptation which gives rise to preferential AR-45 nuclear expression, thereby ensuring appropriate growth for males in the presence of maternal asthma; however, this adaptation disappears when pregnant individuals experience an asthma exacerbation which may be a contributing factor to increased rates of SGA and intrauterine growth restriction (IUGR) in males from uncontrolled asthmatic pregnancies [19].

Declaration of interest

The authors report no conflicts of interest in this work.

Funding

VC was supported by NHMRC SRF APP1136100 and APP1041918. Funding for this project was also provided by MMRI-UQ and the Mater Foundation.

Acknowledgements

The authors would like to acknowledge the valuable contributions made towards the collection and recording of data utilised in this study by the following individuals: Karen Rivers, Nicolette Hodyl, Kate Roberts-Thomson, Annette Osei-Kumah, and Sarah Riley. The authors would also the like to acknowledge the input from the clinical team at the Lyell McEwin Hospital that includes Dr Anil Roy, Dr Bill Jeffries, A/Prof Brian Smith, and Prof Gustaaf Dekker. The authors would also like to acknowledge Dr Luke Selth for intellectual input and the donation of antibodies used in the initial investigation and Dr Thomas Kryza for intellectual input.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.03.012.

References

[1] T.A. Dobbins, et al., Australian national birthweight percentiles by sex and gestational age, 1998-2007, Med. J. Aust. 197 (5) (2012) 291–294.
[2] A.S. Meakin, et al., Review: placental adaptations to the presence of maternal asthma during pregnancy, Placenta 54 (2017) 17–23.
[3] N. Sculthorpe, et al., Androgens affect myogenesis in vitro and increase local IGF-1 expression, Med. Sci. Sports Exerc. 44 (4) (2012) 610–615.
[4] E.R. Cleys, et al., Androgen receptor and histone lysine demethylases in ovine placenta, PLoS One 10 (2) (2015) e0117472.
[5] S. Makieva, P.T. Saunders, J.E. Norman, Androgens in pregnancy: roles in parturition, Hum. Reprod. Update 20 (4) (2014) 542–559.
[6] X. Zhou, Roles of androgen receptor in male and female reproduction: lessons from global and cell-specific androgen receptor knockout (ARKO) mice, J. Androl. 31 (3) (2010) 235–243.
[7] F. de Zegher, et al., Androgens and fetal growth, Horm. Res. 50 (4) (1998) 243–244.
[8] T. Hsu, et al., Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies, Taiwan. J. Obstet. Gynecol. 48 (3) (2009) 5.
[9] K. Sathishkumar, et al., Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia, J. Perinatol. 32 (5) (2012) 328–335.
[10] Y. Li, et al., AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression, Oncogene 31 (45) (2012) 4759–4767.
[11] C. Lu, J. Luo, Decoding the androgen receptor splice variants, Transl. Androl. Urol. 2 (3) (2013) 178–186.
[12] D.G. Hu, et al., Identification of androgen receptor splice variant transcripts in breast cancer cell lines and human tissues, Horm Cancer 5 (2) (2014) 61–71.
[13] I. Ahrens-Fath, et al., Androgen receptor function is modulated by the tissue-specific AR45 variant, FEBS J. 272 (1) (2004) 74–84.
[14] M.B. Stope, et al., Shortened isoforms of the androgen receptor are regulated by the cytoprotective heat-shock protein HSPB1 and the tumor-suppressive microRNA miR-1 in prostate cancer cells, Anticancer Res. 33 (11) (2013) 4921–4926.
[15] R. Hu, et al., Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer, Cancer Res. 69 (1) (2009) 16–22.
[16] V.L. Clifton, et al., Maternal and neonatal outcomes of pregnancies complicated by asthma in an Australian population, Aust. N. Z. J. Obstet. Gynaecol. 49 (6) (2009) 619–626.
[17] G. Rejno, et al., Asthma during pregnancy in a population-based study–pregnancy complications and adverse perinatal outcomes, PLoS One 9 (8) (2014) e104755.
[18] L.E. Grzeskowiak, et al., Patterns, predictors and outcomes of asthma control and exacerbations during pregnancy: a prospective cohort study, ERJ Open Research 2 (1) (2016) 00054-2015-54-2015.
[19] A.S. Meakin, et al., Review: placental adaptations to the presence of maternal asthma during pregnancy, Placenta 54 (2017) 17–23.
[20] V.E. Murphy, et al., A meta-analysis of adverse perinatal outcomes in women with asthma, BJOG 118 (11) (2011) 1314–1323.
[21] Z. Saif, et al., Expression of eight glucocorticoid receptor isoforms in the human preterm placenta vary with fetal sex and birthweight, Placenta 36 (7) (2015) 723–730.
[22] J.S. Horoszewicz, et al., LNCaP model of human prostatic carcinoma, Cancer Res. 43 (4) (1983) 1809–1818.
[23] R.A. Pattillo, G.O. Gey, The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro, Cancer Res. 28 (7) (1968) 1231–1236.
[24] N.A. Hodyl, et al., Sex-specific associations between cortisol and birth weight in pregnancies complicated by asthma are not due to differential glucocorticoid receptor expression, Thorax 65 (8) (2010) 677–683.
[25] N. Harada, et al., Androgen deprivation causes truncation of the C-terminal region of androgen receptor in human prostate cancer LNCaP cells, Cancer Sci. 103 (6) (2012) 1022–1027.
[26] Y. Mitani, et al., Alterations associated with androgen receptor gene activation in salivary duct carcinoma of both sexes: potential therapeutic ramifications, Clin. Cancer Res. 20 (24) (2014) 6570–6581.
[27] R. Azziz, et al., Regulation of extragonadal insulin-like growth factor-binding protein-3 by testosterone in oophorectomized women, J. Clin. Endocrinol. Metab. 79 (6) (1994) 1747–1751.
[28] C.W. Gregory, et al., Androgen receptor up-regulates insulin-like growth factor binding protein-5 (IGFBP-5) expression in a human prostate cancer xenograft, Endocrinology 140 (5) (1999) 2372–2381.
[29] G. Pandini, et al., Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells, Cancer Res. 65 (5) (2005) 1849–1857.
[30] B. Weiss, H. Faus, B. Haendler, Phylogenetic conservation of the androgen receptor AR45 variant form in placental mammals, Gene 399 (2) (2007) 105–111.
[31] T. Hickey, et al., Expression of androgen receptor splice variants in clinical breast cancers, Oncotarget 7 (2015) 17.
[32] Y. Zhan, et al., Interplay between cytoplasmic and nuclear androgen receptor splice variants mediates castration resistance, Mol. Canc. Res. 15 (1) (2017) 59–68.
[33] W. Fan, et al., Insulin-like growth factor 1/insulin signaling activates androgen signaling through direct interactions of Foxo1 with androgen receptor, J. Biol. Chem. 282 (10) (2007) 7329–7338.
[34] C. Porta, C. Paglino, A. Mosca, Targeting PI3K/Akt/mTOR signaling in cancer, Front Oncol 4 (2014).
[35] J. Karar, A. Maity, PI3K/AKT/mTOR pathway in angiogenesis, Front. Mol. Neurosci. 4 (2011).
[36] S. Floyd, et al., The insulin-like growth factor-I–mTOR signaling pathway induces the mitochondrial pyrimidine nucleotide carrier to promote cell growth, Mol. Biol. Cell 18 (9) (2007) 3545–3555.
[37] N. Jansson, et al., Activation of placental mTOR signaling and amino acid transporters in obese women giving birth to large babies, J. Clin. Endocrinol. Metab. 98 (1) (2013) 105–113.
[38] S. Roos, T.L. Powell, T. Jansson, Placental mTOR links maternal nutrient availability to fetal growth, Biochem. Soc. Trans. 37 (Pt 1) (2009) 295–298.
[39] D.R. Clemmons, IGF binding proteins and their functions, Mol. Reprod. Dev. 35 (4) (1993) 368–374 discussion 374-5.
[40] D.A. Salih, et al., Insulin-like growth factor-binding protein 5 (Igfbp5) compromises survival, growth, muscle development, and fertility in mice, Proc. Natl. Acad. Sci. U. S. A. 101 (12) (2004) 4314–4319.
[41] N. Miyakoshi, et al., Evidence that IGF-binding protein-5 functions as a growth factor, J. Clin. Investig. 107 (1) (2001) 73–81.