Sexual Dimorphism in Testosterone Programming of Cardiomyocyte Development in Sheep

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**Sexual dimorphism in testosterone programming of cardiomyocyte development
in sheep**

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Abstract:

Perturbed in-utero hormone milieu leads to intrauterine growth retardation (IUGR), a known risk factor for left ventricular (LV) dysfunction later in life. Gestational testosterone (T) excess predisposes offspring to IUGR and leads to LV myocardial disarray and hypertension in adult females. However, the early impact of T excess on LV programming and if it is female-specific is unknown. LV tissues were obtained at day 90 gestation from days 30-90 T-treated or control fetuses (n=6/group/sex) and morphometric and molecular analyses were conducted. Gestational T treatment increased cardiomyocyte number only in female fetuses. T excess up-regulated receptor expression of insulin and insulin-like growth factor. Furthermore, in a sex-specific manner, T increased expression of Phosphatidylinositol 3-kinase (PI3K) while down regulating phosphorylated mammalian target of rapamycin (pmTOR) /mTOR ratio suggestive of compensatory response. T excess 1) upregulated atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), markers of stress and cardiac hypertrophy 2) upregulated estrogen receptors1 (ESR1) and 2 (ESR2) but not in androgen receptor (AR). Thus, gestational T excess upregulated markers of cardiac stress and hypertrophy in both sexes while inducing cardiomyocyte hyperplasia only in females, likely mediated via insulin and estrogenic programming.

Introduction

CVD remains the leading cause of death worldwide, with a prevalence rate of 49.2% increasing with age in males and females [1]. Pathological cardiac remodeling and left ventricle hypertrophy (LVH), typically preceding progression to heart failure, account for

a substantial portion of CVD morbidity and mortality [2-4]. Despite significant advances in identifying CVD risk factors and their therapeutics, the morbidity and mortality from CVD remain high [5, 6]. Of note, sex differences exist in both prevalence and burden of CVD. Despite it being the leading cause of death in both men and women, men have a higher age-adjusted rate of CVD mortality [7]. Men are more prone to pathological LV remodeling, and heart failure outcomes are worse in men compared to women [8]. Furthermore, with aging, there is a sex-specific decrease in cardiomyocyte number and an increase in myocytes size, more pronounced in males than in females [9]. Of relevance, alteration in sex steroid levels impacts cardiovascular function in a sex-specific manner [10]. While estrogen is considered cardioprotective in women [10], androgens are linked to CVD risk in both sexes [11, 12]. However, the underlying molecular mechanisms and sex-specific impact of CVD-related pathological cardiac remodeling and associated morbidity and mortality are not well understood.

Epidemiological data points to obesity [13], hyperglycemia [14], hypertension [15], and physical inactivity [16] as major CVD risk factors that contribute to morbidity and mortality [17]. Substantial evidence from human, animal, and epidemiological studies indicate that early life insults in-utero lead to IUGR, adversely program the cardiovascular system, thereby predisposing to CVD later in life [18-20], including LVH [21, 22]. Interestingly, many of these insults are associated with androgen excess [23, 24], a well-known risk factor for the development of pathological LVH and CVD in offspring [25-27]. For instance, a recent epidemiological study noted that an elevated third-trimester androgen level was positively correlated with a 4.84-fold increased risk of hypertension in female offspring [28]. Moreover, higher fetal testosterone (T) levels in

late pregnancy were also associated with higher blood pressure in young adults [29]. Similarly, offspring of hyperandrogenic women with PCOS were reported to have alteration in blood pressure, left ventricular dilation, and increased carotid intima thickness [30].

Animal studies have corroborated the adverse impact of excess prenatal T exposure on cardiovascular health. Using our well-characterized sheep model of prenatal T excess [31], we previously found exposure to excess T during fetal life results in mild hypertension and pathological cardiac remodeling in adult female offspring [32, 33] (the impact in male offspring remain to be determined). In rodent studies, where both sexes were investigated, prenatal T excess induced hypertension in both males and females with greater effect seen in males [34].

While the detrimental effects of exposure to excess T during fetal life on cardiometabolic phenotype are well-validated in several species [35-37], the early perturbations leading to pathological cardiac remodeling and LVH are not well understood. Relative to mediators of cardiovascular programming, estrogen and T play a key role in cardiac growth [38, 39], exerting their action by binding to their cognate estrogen and androgen receptors that are present on cardiomyocytes of several species, including human [40-42]. Inappropriate androgen and estrogen levels enhance progression to heart failure [42], and T can mediate cardiac hypertrophy in cultured cardiomyocytes [39]. While the exact molecular mechanisms underlying the mediation of cardiac hypertrophy by T are poorly understood, studies suggest T may mediate hypertrophy via activation of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway [43]. Both T and estrogen also activate the phosphoinositide 3-kinase

(PI3K)–AKT signaling pathway that is also activated by insulin and insulin-like growth factor 1 (IGF1) [40, 44]. In this context, gestational T excess from days 30-90 of gestation was found to induce maternal hyperinsulinism [45], increase fetal estrogen [46], and reduce fetal IGF-1 levels at day fetal day 140 [47] culminating in IUGR [48]. Considering low birth weight as a risk factor for the development of CVD [49] and prenatal T excess leads to low birth weight in both sexes [48], both males and females are at risk of adverse cardiac programming. A recent study by Jonker et al. found prenatal T treatment of sheep from gestational days 30-59 reduced cardiomyocyte maturation and proliferation at birth more so in males than females offspring [50], thus providing some support of sexually dimorphic cardiac programming. However, the adult cardiovascular phenotype with 30-59 day window of T exposure used in Jonker's study is not characterized. Importantly, whether the extended exposure period from days 30-90 of fetal life that results in adult hypertension in the female [32] leads to a more severe cardiomyocyte phenotype early in life is also unclear.

Considering CVD outcomes differ between men and women and the limited information available on the sexually dimorphic impact of prenatal T excess, especially the early cardiac perturbations that contribute to adverse programming of LVH, the objective of this study was to investigate the impact of exposure to excess T from days 30-90 of gestation on sex-specific early cardiac effects using the sheep model. Because T fetuses are exposed to high androgen, estrogen, insulin, and altered IGF during fetal life, we hypothesized that the impact of prenatal T excess on LV remodeling involved early changes in androgen/estrogen/insulin/IGF signaling pathways leading to alterations in cardiomyocyte morphology.

Results

Impact of gestational T excess on fetal body and heart weights:

Analyses of body weights revealed no sex ($P=0.52$), treatment ($P=0.93$), or sex by treatment interaction ($P=0.18$). Two-way ANOVA showed no significant sex ($P=0.7$) or treatment ($P=0.56$) effect in heart weights, although a trend ($P=0.07$) for sex by treatment interaction was evident. This trend in interaction was reflected as a large magnitude decrease in heart weight (Cohen's $d=0.85$) in T females compared to CON females as opposed to a medium magnitude increase (Cohen's $d=0.65$) in heart weight in T male fetuses compared to CON fetuses (**Fig. 1B**). However, analysis of heart weight to body weight ratio revealed no effect of sex ($P=0.92$), treatment ($P=0.28$), or sex by treatment interaction ($p=0.15$) (**Fig. 1C**).

Impact of gestational T excess on cardiomyocyte number, diameter, and collagen

content: Two-way ANOVA showed significant sex, treatment, and sex by treatment interaction in cardiomyocyte number. T-treated females exhibited a significant increase in cardiomyocyte number compared to control females (**Fig.2A, 2B**). Cohens effect size analysis also revealed a large magnitude increase in cardiomyocyte number in T-treated female fetuses (Cohen's $d =2.93$) but not male (Cohen's $d =0.02$). In contrast, two-way ANOVA showed a significant sex effect but no treatment ($P =0.11$) or sex by treatment interaction ($P=0.17$) in cardiomyocyte diameter (**Fig.2C**). There was no significant impact of sex, treatment, and sex by treatment interaction in collagen accumulation (**Fig.3A and B**).

Impact of gestational T excess on steroid receptors

Two-way ANOVA of ESR1 found no sex effect ($P = 0.46$), a significant T treatment effect, and no significant sex by treatment interaction ($P = 0.38$). Cohen's effect size analysis revealed a large magnitude increase in T males (Cohen's $d = 0.99$) and T females (Cohen's $d = 1.73$) relative to their sex-matched controls (**Fig. 4A**). Similarly, while there was no significant sex difference in ESR2 expression ($P = 0.28$), there was a significant T treatment effect. Consistent with the lack of sex by treatment interaction, Cohen's effect size analysis revealed an increase in ESR2 expression in T female fetuses (Cohen's $d = 1.6$) as well as the T-treated males (Cohen's $d = 1.03$ (**Fig. 4B**) relative to their sex-matched controls. In contrast to effects of T treatment on ESR1 and ESR2 expressions, there were no significant sex ($P = 0.8$), treatment ($P = 0.6$), or interaction effects ($P = 0.8$) relative to AR expression (**Fig. 4C**).

Impact of gestational T excess on markers of LV hyperplasia and stress.

Two-way ANOVA of α -MHC gene expression showed no sex effect ($P = 0.2$), a significant T treatment effect, but no significant sex by treatment interaction ($P = 0.31$). Relative to treatment effects, Cohen's effect analyses also revealed a large increase in T-treated males (Cohen's $d = 1.15$) and T females (Cohen's $d = 1.01$) compared to their sex-matched controls (**Fig. 5A**). Similarly, as opposed to a lack of sex effect but a treatment effect with α -MHC, there was a significant sex effect but no treatment ($P = 0.44$) effect with β -MHC expression. There was also no significant sex by treatment interaction with both α -MHC ($P = 0.31$) and β -MHC ($P = 0.21$) expression (**Fig. 5B**). There

were no sex ($P = 0.23$), treatment ($P = 0.54$) or sex by treatment interaction ($P = 0.86$) in β -MHC/ α -MHC expression ratio (**Fig.5C**).

ANP gene expression showed significant sex and treatment effect, although sex by treatment interaction was not significant ($P = 0.10$). Cohens effect size analyses revealed a large effect size difference in ANP expression between control and T-treated females (Cohen's $d = 1.44$) that reached significance, but only a medium magnitude difference (Cohen's $d = 0.73$) between control and T-treated males that did not achieve significance (**Fig.5D**). In contrast, BNP gene expression showed no sex effect ($P = 0.21$) although there was a significant treatment effect with Cohens effect analysis revealing a large effect size increase in T-treated males (Cohen's $d = 1.27$) and a medium effect size increase (Cohen's $d = 0.5$) in T-treated females (**Fig.5E**). There was no sex by treatment interaction ($P = 0.22$).

Impact of gestational T excess on insulin signaling mediators.

Two-way ANOVA showed a trend in sex ($P = 0.058$), a significant treatment, and a trend in sex by treatment interaction ($P = 0.067$) in IR gene expression. Cohens effect size analyses revealed a large magnitude increase (Cohen's $d = 1.40$) in IR expression in T-treated female fetuses that achieved statistical significance by post hoc analyses and only a medium magnitude increase (Cohen's $d = 0.63$) in T-treated males relative to corresponding controls that did not achieve significance (**Fig.6A**). IRS1 gene expression showed no significant sex ($P = 0.65$), treatment ($P = 0.45$) or sex by treatment ($P = 0.69$) effects (**Fig.6B**). In contrast, IRS 2 showed no significant sex ($P = 0.11$) or treatment ($P = 0.29$) effect, although a trend in sex by treatment effect was evident (**Fig.6C**). Cohens

187 effect analyses revealed a large effect size increase (Cohen's $d = 1.07$) only in T-treated
188 females compared to controls that did not reach statistical significance due to the small
189 sample size. Similar to IRS2, PI3K expression also showed no sex ($P = 0.22$) or
190 treatment ($P = 0.11$) effect but revealed significant sex by treatment interaction. The
191 directionality of T treatment effects differed between sexes, with Cohens effect size
192 analysis revealing a large magnitude increase (Cohen's $d = 1.11$) in T females as
193 opposed to a large magnitude decrease (Cohen's $d = 1.11$) in T-treated males compared
194 to their respective controls (**Fig.6D**). There were no significant sex ($P = 0.91$), treatment
195 ($P = 0.54$) or sex by treatment interaction ($P = 0.88$) with AKT gene expression (**Fig.6E**).
196 In contrast, there was a trend for a sex ($P = 0.07$) and treatment ($P = 0.07$) effect with
197 mTOR gene expression although the interaction ($P = 0.19$) was not significant. The trend
198 for treatment effect was reflected with Cohens effect size analyses revealing a large
199 effect size increase in mTOR in T-treated females (Cohen's $d = 1.01$) and a medium
200 effect size increase in T-treated males (Cohen's $d = 0.60$) relative to their controls
201 (**Fig.6F**). Directionality of changes in GLUT4 expression followed that of mTOR with a
202 trend for a sex effect ($p = 0.08$), significant treatment effect, and no sex by treatment (P
203 $= 0.64$) effect. Cohens analyses also revealed a large magnitude increase in GLUT4 in
204 T-treated females (Cohen's $d = 0.88$) as well as T males (Cohen's $d = 1.68$) relative to
205 corresponding sex-matched controls (**Fig.6G**).
206 Changes in protein expression of pmTOR showed no significant sex ($P = 0.64$),
207 treatment ($P = 0.27$) or sex by treatment interaction ($P = 0.48$) in the LV (**Fig.7A-A**). In
208 contrast, while there was no significant sex ($P = 0.88$) or treatment ($P = 0.80$) effects, a
209 significant sex & treatment interaction was evident with protein expression of total

mTOR (**Fig.7A-B**). Post hoc analyses showed a significant reduction in mTOR protein expression in T-treated males ($p=0.026$) and a trend for an increase ($p=0.058$) in T-treated females compared to their control groups. Cohens effect size analyses also revealed a large effect size difference between control and T-treated males (Cohen's $d=2.97$) and control and T-treated females (Cohen's $d=1.30$) (**Fig.7A-B**). Ratio of pmTOR/mTOR showed no sex effect ($P=0.16$), a trend for treatment effect ($P=0.096$) and a significant sex by treatment interaction. Post hoc analyses showed a significant reduction in protein expression in T-treated females only ($p=0.036$). Cohens effect size analyses revealed a large effect size difference only between control and T-treated females (Cohen's $d=1.56$) (**Fig.7A-C**). As opposed to lack of sex effect with mTOR, two-way ANOVA showed a significant sex effect ($P=0.006$) in GLUT4 protein expression but no treatment ($P=0.37$) or sex by treatment interaction ($P=0.78$) (**Fig.7A-D**). Examination of GSK-3 β protein expression downstream of mTOR in the insulin signaling pathway followed by two-way ANOVA showed a significant sex and treatment effects but no sex by treatment interaction effect in pGSK-3 β protein expression with Cohen's effect size analyses revealing a large magnitude increase in T-treated females (Cohen's $d=0.98$) and a medium magnitude increase (Cohen's $d=0.73$) in T-treated males compared to their sex-matched controls (**Fig.7B-A**). There were no significant sex ($P=0.9$), treatment ($P=0.9$) or sex by treatment interaction ($P=0.53$) with total GSK-3 β protein expression (**Fig.7B-B**). Ratio of pGSK-3 β / GSK-3 β showed a significant sex effect but no treatment ($P=0.19$) or sex by treatment interaction $P=0.52$) (**Fig.7B-C**).

Impact of gestational T excess on IGF1 signaling

Two-way ANOVA of IGF1 expression showed no sex difference ($P = 0.55$), a trend for a treatment effect ($P = 0.05$), and no sex by treatment interaction effect ($P = 0.51$). Reflective of increased trend, Cohen's effect analyses revealed a large effect size increase in IGF1 in T females (Cohen's $d = 0.97$) as well as T-treated males (Cohen's $d = 1.06$) compared to the corresponding control (**Fig.8A**). IGF1R expression showed a trend for a sex effect ($P = 0.06$), a significant treatment effect, and a trend for sex by treatment interaction ($P = 0.08$). Cohens effect analyses revealed a large effect size increase in T-treated females (Cohen's $d = 1.64$) that achieved statistical significance and a large effect size increase T-treated males (Cohen's $d = 1.31$) that did not achieve statistical significance (**Fig.8B**).

Discussion

Major findings from the present study include the impact of gestational T excess in inducing: 1) Significant increase in cardiomyocytes number in female but not male day 90 fetuses indicative of sex-specific effects 2) Significant increase in expression of IR and IGFR with T treatment with higher levels in T -treated female fetuses compared to controls and female-specific up-regulation in expression of PI3K 3) Significant increases in ANP and BNP, markers of cardiac stress and hyperplasia with T treatment in both sexes 4) up-regulation of ESR1 and 2 but not AR in the fetal LV of both sexes suggestive of a role for estrogenic programming. Overall, findings from this study provide evidence that gestational T excess alters the developmental trajectory of LV

morphology and molecular signaling mechanisms mediating cardiac growth during early fetal life in a sex-specific manner.

Impact of gestational T excess on fetal myocardial morphology:

During fetal development, cardiomyocytes grow initially by cellular hyperplasia, with cardiomyocytes initiating the transition to hypertrophy towards the end of gestation [51]. Changes in in-utero hormonal milieu during the critical period of cardiac development have the potential to alter the trajectory of the developing cardiomyocytes. Studies with animal models of in-utero perturbation of hormonal milieu have documented adverse programming of cardiomyocyte morphology [52-55]), the impact of which differs depending on the timing of exposure, fetal sex and the developmental time point of the investigation. For instance, studies of Jonker et al. noted exposure to T excess between day 30-59 days of fetal life reduced cardiomyocyte maturation and proliferation in day 135 sheep fetuses, impacting males more than females [50]. In contrast, exposure to T from days 30-90 gestation (this study) led to female-specific cardiomyocyte hyperplasia at day 90 of fetal life. Furthermore, while the Jonker study found reduced heart weight in both sexes at day 135 gestation, studies detailed in this investigation found diametrically opposite outcomes in male and female fetuses on day 90 of fetal life reflected as decreased heart weight in females as opposed to an increase in heart weight in males. These differences between studies may be a function of the time point in fetal life studied (Day 135 in Jonker et al. and Day 90 in present study), duration of T treatment (Day 30-59 in Jonker et al. vs. Day 30-90 in the present study) or a function of the sheep breed used (Polypay breed in Jonker et al. and Suffolk in the

present study). To what extent the cardiac hyperplasia evident in day 90 Suffolk female fetuses in the present study contributes to the adult cardiovascular phenotype [32, 33] is unclear. Furthermore, since cardiomyocyte hyperplasia/hypertrophy can be adaptive vs maladaptive response to stimuli [55], the increase in cardiac stress markers (BNP and ANP) in our study suggests early fetal life maladaptive LV response. Functional studies addressing sex differences in postnatal cardiovascular function will assist in determining if the molecular changes seen in fetal life in the current study are adaptive vs. maladaptive programming.

Impact of gestational T excess on the fetal myocardium: underlying mechanisms

Alterations in steroidal and metabolic signaling pathways resulting from gestational testosterone treatment could be contributing to the changes in cardiomyocyte morphology and molecular changes seen in this study. Previously, we found gestational T treatment, in addition to increasing T levels, induced maternal hyperinsulinemia [45], increases fetal levels of estrogen in both male and female fetuses [46], and transient increases in plasma IGF-I and IGFBP-3 concentrations at fetal day 90 [47] providing evidence in support of the potential for impact on cardiomyocyte via androgenic, estrogenic, insulin/IGF signaling pathways.

Gestational T excess on fetal myocardial steroidal signaling:

Despite the presence of both estrogen and androgen receptors in mammalian cardiomyocytes [40], the role of estrogen/androgen signaling pathways in cardiomyocyte proliferation and maturation is not well understood. Systemic sex specific changes in the steroids milieu may modulate myocardial estrogen and androgen

receptor expression with their activation eliciting both genomic and non-genomic actions [40]. Non-genomic action of both estrogen and androgen can be via activation of PI3K/AKT signaling [40]. Our earlier studies have shown increases in fetal estrogen levels in day 90 fetuses [46] following exposure to T excess supportive of the potential for mediation via estrogenic signaling pathways. Although the potential for involvement of androgenic or estrogenic pathways in the activation of PI3K in female fetuses exists, our finding of upregulation of both estrogen receptor isoforms (ESR1 and ESR2) coupled with lack of changes in expression of AR suggests that effects of gestational T excess are likely mediated via estrogenic rather than androgenic signaling pathways. In fact, there is some evidence for adverse fetal molecular programming of LV with exposure to an estrogen mimetic chemical in-utero; gestational treatment with estrogenic mimetic bisphenol A (BPA) altered fetal LV transcriptome in rhesus monkey involved with cardiac pathologies, including myosin heavy chain 6 (Myh6), a gene coding for α MHC [56]. In agreement with this finding, an alteration in α MHC gene involved in the contractile function of the heart [57] was evident in gestational T-treated fetuses (this study). Coupled with upregulation of ESR1 and ESR2 in the LV these findings support the potential for estrogenic signaling in cardiomyocyte development.

Gestational T excess on fetal myocardial Insulin and Insulin-like growth factor signaling:

Insulin signaling: Insulin is one of the key mediators of fetal growth. Perturbation in insulin homeostasis and organ-specific signaling leads to insulin resistance, a known risk factor for pathological cardiac remodeling, including LVH and CVD [58, 59]. For

instance, maternal diabetes mellitus predisposes fetal myocardial hypertrophy [60, 61]. Fetal hyperinsulinism and upregulation of insulin receptors have been shown to lead to cardiomyocytes proliferation and hypertrophy in infants [62, 63]. The finding from the present study documenting a female- specific increase in PI3K and trend in the increase of IRS is consistent with the involvement of insulin signaling pathway in the sex-specific mediation of cardiac hyperplasia in the female. Our earlier finding that gestational T excess leads to maternal hyperinsulinemia [45] supports enhanced insulin signaling at the ligand level as well. It is well recognized that PI3K/mTOR pathway downstream of IR activation is a key mediator of cardiac growth and adaptation to cardiac stress [3, 64, 65]. The decrease in the pmTOR/mTOR protein expression ratio in the female despite an increase in cardiomyocyte hyperplasia may represent a compensatory response to prevent ongoing cardiac perturbation. Lack of change in protein expression of GLUT 4 under basal condition suggests that early cardiomyocyte changes seen in LV may not alter myocardial insulin sensitivity at this early stage of programming.

IGF-1 signaling: Parallel with insulin, the IGF system also plays a key role in cardiomyocytes proliferation and maturation during cardiac development [66]. IGF-1 induces its action by binding to IGF1R and activating downstream signaling pathways including activation of PI3K/mTOR [67]. Involvement of the IGF-1 signaling pathway in cardiac development is evident from both in-vitro and in-vivo animal studies. Fetal sheep infused with IGF-I exhibit increased size and binucleated cardiac myocytes in females compared to males [68]. Similarly, activation of IGF-1R resulted in stimulation of myocyte proliferation but not cellular hypertrophy in-vitro in the rat [69]. As such the increase in IGF-1 and IGF-1 R expression at fetal day 90 following gestational T excess

and a trend toward higher expression in female fetal LV suggests that IGF-1 signaling could act in concert with insulin in mediating sex-specific cardiomyocyte hyperplasia in female fetuses. In the context of involvement of the IGF axis, prenatal T excess increases IGF-1 levels in day 90 female fetuses (male levels unknown) [47]. The finding of Jonker et al. that T exposure from days 30-59 gestation decreases IGF-1 levels in day 135 fetus [50] while at odds with our finding may be a function of differences in duration of excess T exposure, the breed of sheep used and differences in gestational age studies.

Sexually dimorphic effects with gestational T excess:

Sexually dimorphic LV morphology and molecular signaling in early fetal life: Our findings revealed sex-specific differences in cardiomyocyte diameter with an increase in males compared to D90 female fetuses. Although sex-specific differences in cardiomyocyte size are not known during mid-gestation for ovine fetuses, evidence points to an increase in cardiomyocyte size in near-term females compared to males [68], opposite of what was seen in our study during day 90 fetal life. Because sheep are precocial with primordial ovarian follicular differentiation occurring beginning day 90 of life and mature follicles differentiating in late gestation fetuses [70], to what extent does the increase in estrogen in near term fetuses contribute to this female specific increase in cardiomyocyte diameter is unclear. The increase in GLUT 4 in male LV compared to females is suggestive of increased reliance of male LVs on glucose for energy metabolism during mid-gestation.

Apart from sex differences in cardiac morphology and metabolic molecular markers, we also noted sex differences in proteins involved in cardiac contractile machinery. Female fetal LV expresses higher β -MHC compared to male fetuses. Since β -MHC is less reliant on ATP compared to α -MHC [57], female hearts are likely to be less reliant on ATP for contraction compared to male hearts. Lack of differences in estrogen and androgen receptor expression between male and female fetal LV suggests inputs from sex steroidal signaling in the heart are comparable at early fetal life. As such the sex differences in LV molecular phenotype seen in fetal life may be a function of their metabolic milieu.

Sexual dimorphic effect of T on fetal LV morphology and molecular signaling in early gestation (Figure 9).

Apart from the sexually dimorphic trend in heart weight proportionate to the bodyweight between male and female fetuses and female-specific increase in cardiomyocyte hyperplasia with prenatal T excess, sex differences in molecular markers responsible for such differences were evident. For instance, mTOR that is central to cardiac growth [64, 65] was downregulated in LV of T female fetuses at this early gestational time point, potentially signifying a compensatory response to overcome the hyperplasia in the female. Of importance, sex-specific alteration in cardiomyocyte size and numbers during fetal life is a reported risk factor for cardiovascular disease later in life [55].

Female-specific upregulation in markers of insulin signaling, namely PI3K and trend in IRS2 as well as higher magnitude increase in IR and IGFR suggests that insulin/IGF signaling pathway may be major contributors in the sexually dimorphic cardiomyocyte

hyperplasia. To what extent the sex-specific differences in LV morphological and molecular phenotype seen in the heart of T fetuses would translate to adult cardiovascular differences is unknown. While information is available on the impact of fetal exposure to T excess on cardiac morphology and molecular markers in adult females following gestational day 30-90 T treatment [33], data is lacking in males. Nonetheless, a large body of evidence prevails that points to sex differences in cardiovascular physiology and pathophysiology [71-73], such as higher left ventricular (LV) mass in boys compared to girls [73] during the peripubertal period and women having smaller hearts compared to men [74]. As such, it is conceivable sex differences in cardiac phenotype will likely be the outcome in this model as well.

Translational relevance:

Animal models have been long used to gain mechanistic insights into the developmental origin of diseases. Sheep are excellent large animal models to study the developmental origin of adult cardiovascular pathologies. Similar to humans, sheep are precocial species, and most organ system differentiation occurs prior to birth [70]. Of relevance to this manuscript, cardiomyocyte differentiation during fetal development in sheep is also similar to humans [51, 53, 75]. Importantly, sheep have emerged as a suitable widely used model for cardiovascular studies due to the similarity in the cardiovascular system between humans and sheep [76, 77]. Prenatal T-treated sheep have served as excellent model systems to understand adverse programming of multiple metabolic organ systems [31, 78-81]. Furthermore, considering prenatal T excess leads to IUGR [48] and the female offspring recapitulates the PCOS phenotype [82] both risk factors

for cardiovascular dysfunction [30, 83], our findings in the present study have translational relevance relative to the developmental origins of CVD in offspring. Determining the early histological and molecular alteration in the LV of both males and females from prenatal T excess allows designing sex-specific early interventions to prevent CVD onset.

In conclusion, findings of the present study demonstrate sex-specific effects of gestational T excess between days 30-90 of gestation on the cardiac phenotype. Furthermore, the sex-specific programming is likely secondary to perturbation in both estrogen and insulin signaling pathways collectively. These findings are supportive of the role of androgen excess to serve as early biomarkers of CVD and could be critical in identifying therapeutic targets for LV hypertrophy and predict long-term CVD.

Methods

Experimental animals, prenatal T-treatment, and tissue collection

All experimental animal procedures were approved by the University of Michigan Animal Care and Use Committee. All methods were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The study was carried out in compliance with ARRIVE guidelines. Animals were bred at the University of Michigan Sheep Research Facility (Ann Arbor). The date of mating was determined by paint markings left by the ram on the rumps of ewes. Pregnant ewes were assigned randomly between control (Control) and T-treatment (T- Treated) groups adjusting for body weight (mean $\pm$ SEM: control 83.41 ± 31 vs. T- Treated 81.64 ± 4.2 kg) and body score (control 2.55 ± 0.17 vs. T- Treated 2.76 ± 0.16). For generating T-treated fetuses, pregnant ewes were injected with testosterone propionate (T) suspended in 2 ml corn

oil, 100 mg i.m. twice weekly (~1.2 mg/kg; Sigma-Aldrich, St. Louis, MO) from gestational day 30 to 90; term: 147 days (**Fig.1A**). Controls received 2 ml of corn oil vehicle. At the end of T treatment (mean $\pm$ SEM: 91.9 ± 0.2) of gestation, ewes were euthanized as previously described [46]. Briefly, sedation was induced with 20–30 ml of pentobarbital i.v. (Nembutol Na solution, 50 mg/ml; Abbott Laboratories, Chicago, IL), and anesthesia was maintained with 1-2% halothane (Halocarbon Laboratories, River Edge, NJ). The gravid uterus was exposed through a midline incision, and male and female fetuses were collected, and the hearts removed. The dam was the experimental unit with only one randomly selected male or female fetus used from each dam if there were more than one fetus. Fetal body and heart weights were recorded. LV tissues were separated, snap-frozen, and stored at -80°C until utilized for mRNA and western blot analysis or fixed in formaldehyde in PBS pH 7.4 and paraffin-embedded for molecular and histological analyses.

RNA extraction and quantitative RT PCR analysis

Real-time PCR (RT-PCR) was used to examine gene expression in the myocardial tissue (n= 6 Controls and 6 T-Treated males and females). LV tissue was used to extract total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA). Following isolation, RNA was treated with DNase to digest DNA and purified using the RNAeasy kit (Qiagen, Germantown, MD) to obtain high-quality RNA according to the manufacturers' instructions. RNA quality was determined spectrophotometrically using NanoDrop (Thermo Fisher Scientific, Waltham, MA) by measuring the OD260/280 ratio with a ratio of 2.0 indicative of good quality RNA. cDNA was prepared from one μg of RNA using

the SuperScript VILO cDNA synthesis kit (Invitrogen). Considering steroids can modulate cardiomyocyte development [40], T can be aromatized to estrogen and prenatal T excess leads to fetal T and estradiol excess [46], the sex-specific effects of gestational T excess on ESR1, ESR2, and AR were determined. Since gestational T excess induces maternal hyperinsulinemia [84], and has an impact on the fetal IGF system [47], the effects of prenatal T excess on insulin signaling pathway (IR, insulin receptor substrate 1(IRS1), IRS2, PI3K, glucose transporter protein type-4 (GLUT4), AKT, mTOR, IGF1, and IGF1 receptors (IGF1R)) were examined. In addition, the effects of prenatal T excess on LV hypertrophy and stress mediators (alpha-myosin heavy chain (α -MHC), beta-myosin heavy chain (β -MHC), ANP, and BNP were also examined. The mRNA levels were measured using A SYBR Green-based QRT-PCR assay and performed using a Bio-Rad IQ5 Real-time-PCR Reaction System (Bio-Rad Laboratories Inc., Hercules, CA). All primer sequences for genes investigated were previously published and are shown in **Table 1**. Each gene was tested in triplicate, averaged, and the expression was determined after normalization with an expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative amount of each transcript (in fold) was measured by using the $\Delta\Delta CT$ method [85].

Protein extraction and western blot analysis

Frozen LV tissues were homogenized in radioimmunoprecipitation assay buffer (Pierce RIPA buffer; Thermo Scientific) containing Protease inhibitors (Thermo Scientific) and phosphatase inhibitor (Thermo Scientific). The homogenized extracts were centrifuged

at 10000g for 15 minutes at 4°C, and the whole-cell protein extract was used for the analysis. Equal amounts of protein (15µg) were resolved on SDS-PAGE and transferred into a nitrocellulose membrane (Bio-Rad). Membranes were incubated in blocking buffer (5% nonfat milk diluted in Tris-buffered saline) for 1 hr at room temperature and incubated overnight at 4°C with primary antibodies (all previously published and are shown in **Table 2**). Primary antibodies including mTOR, P-mTOR, glycogen synthase kinase-3β (GSK3β), P-GSK3β, GLUT 4, and the housekeeping gene GAPDH were obtained from Cell Signaling Technology (Danvers, MA). Levels of phosphorylated and total forms of proteins, as well as the corresponding loading controls, were determined in the same membrane after stripping and reblotting. Samples from all experimental groups were distributed through two SDS-PAGE gels that were run under the same conditions at the same time three times. Protein was visualized using enhanced chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Scientific), and the density of bands was quantified using the ImageJ software (National Institutes of Health). The specificity of the antibodies was confirmed by visualization of protein bands of the correct size.

Cardiomyocyte morphometry

LV tissues were collected and fixed in formalin in PBS pH 7.4 for 48 hours, dehydrated with ethanol, cleared in xylene, and embedded in paraffin. Tissue sections were cut at 5 µm. To determine cardiomyocyte number and diameter, two LV sections per animal separated by 100 µm were stained with hematoxylin and eosin (H&E). The number and diameter of cardiomyocytes were quantified by measuring the short axis of nucleated

transverse sections from six randomly chosen images per animal as previously described [33]. For each image three counting frames (50.10 x 50.10µm) were quantified by an experimenter blinded to the treatment groups with ~300 cardiomyocytes per animal measured. Images were acquired by Leica DM 1000 LED microscope at 63X magnification and quantified using Image J software. To assess development of fibrosis in LV tissues, 3 sections 100 µm apart were stained with Masson's trichrome staining (Abcam, ab150686) according to the manufacturer's instructions. Fibrotic tissue quantification was performed as previously described [86]. They were imaged on Zeiss microscope and analyzed using Image J software (NIH, Bethesda, MD).

Statistical Analysis

After testing for homogeneity of variance, data were log-transformed when needed and analyzed using two-way ANOVA with fetal sex (males; female), treatment (T; CON), and their interaction as the main effects. Statistical outliers were excluded from the analysis using Grubbs' test (<https://www.graphpad.com/quickcalcs/grubbs1/>). Analyses were performed using GraphPad Prism (Prism 9.0, GraphPad Software, San Diego, CA). Data are presented as mean ± SEM and differences were considered significant at $P \leq 0.05$, tendencies at $P \leq 0.10$. A post hoc test using Bonferroni's multiple comparisons analysis was performed when there was either a significant ($P < 0.05$) effect or a trend towards significance ($P \leq 0.10$) in sex by treatment interaction was evident. Additionally, due to the small sample size, data were also analyzed by Cohen's effect size analyses

[87]. Cohen's d value of 0.5-0.8 and 0.8 represent medium and large effect size differences between the T- treated and control groups.

Data availability

All data from the current study are available from the corresponding author upon reasonable request.

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Author Contributions

AG carried out all the measures detailed in this study, carried out the statistical analyses, and participated in the writing of the manuscript. VP was involved in the conceptual development of the study, participated in the writing of the study. AV conceived the study, directed the execution of all measures, participated in the writing of the manuscript.

Competing interests

The authors declare no competing interests.

Figure 1. (A) Schematic diagram of experimental design. Pregnant sheep were used to produce day 90 males and female fetuses treated with either CON or T. At endpoint day 90 of gestation, fetuses were necropsied, and heart LV tissues were harvested either flash frozen and kept in -80 for molecular analysis or fixed for histological analysis. (B) Fetal heart weight. (C) Fetal heart weight to body weight ratio at day 90 of gestation. Values are Mean $\pm$ SEM. (n=6/sex/group). Data analyzed by two-way ANOVA. * $P \leq 0.05$. Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large).

Figure.2. (A) Representative LV sections stained with H&E at 90 days of gestation. (B) Quantification of number of cardiomyocytes in the LV tissues. (C) Diameter of cardiomyocytes. Values are presented as mean $\pm$ S.E.M. (2 sections/animal; 3 images per section); (n= 6). Data analyzed by two-way ANOVA. * $P \leq 0.05$, Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large).

Figure 3. (A). Representative LV sections stained with Masson's trichrome at 90 days of gestation. (B) Quantification of collagen Contents (blue) in the LV tissues. Values are presented as mean $\pm$ S.E.M. (4 sections/animal; 5 images per section); (n= 6/group/sex). Data analyzed by two-way ANOVA. Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large).

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Figure 4. mRNA expression of steroid receptors (A) ESR1, (B) ESR2, and (C) AR in LV tissue. Data are analyzed by two-way ANOVA. Values are means $\pm$ SEM; n=6/group/sex. Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large).

Figure 5. mRNA expression of hypertrophy and stress mediators (A) α -MHC, (B) β -MHC, (C) β -MHC / α -MHC ratio, (D) ANP, and (E) BNP in LV tissue. Data are analyzed by two-way ANOVA. Values are means $\pm$ SEM; $n=6$ / group/sex; * $P \leq 0.05$. Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large).

Figure 6. mRNA expression of insulin signaling pathway (A), IR, (B) IRS1, (C) IRS2, (D) PI3K, (E) AKT, (F) mTOR, and (G) GLUT4 in LV tissue. Data are analyzed by two-way ANOVA. Values are means $\pm$ SEM; $n=6/$ group/sex; * $P \leq 0.05$. Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large).

Figure 7A

Figure 7B

Figure 7A. Protein expression of insulin signaling pathway (A), pMTOR, (B) mTOR, (C) PmTOR/ mTOR ratio, (D) GLUT4 in LV tissue. Data are analyzed by two-way ANOVA. Values are means $\pm$ SEM; $n=6/$ group/sex; * $P \leq 0.05$. Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large). F-C = Female control, M-C = Male control, F-T = Female T-Treated, M-T = Male T-Treated. Full-length blots/gels are presented in supplementary figure 7A.

Figure 7B. Protein expression of insulin signaling pathway (A), pGSK-3 β , (B) GSK-3 β , (C) pGSK-3 β / GSK-3 β ratio in LV tissue. Data are analyzed by two-way ANOVA. Values are means $\pm$ SEM; $n=6/$ group/sex; * $P \leq 0.05$. Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large). F-C = Female control, M-C = Male control, F-T = Female T-Treated, M-T = Male T-Treated. Full-length blots/gels are presented in supplementary figure 7B.

Figure 8. mRNA expression of IGF signaling pathway (A), IGF1 and (B) IGF1R in LV tissue. Data are analyzed by two-way ANOVA. Values are means $\pm$ SEM; $n=6$ /group/sex; $*P \leq 0.05$. Cohen effect sizes ($d = 0.2$, small), ($d = 0.5$, medium), and ($d \geq 0.8$, large).

Figure 9. Proposed schematic of molecular pathways underlying fetal cardiac programming with gestational T excess in cardiomyocyte. Dark Green = Significant increase in expression. Light Green = Trend increase in expression. Yellow = no change, Orange = decreased ratio pmTOR/ mTOR. Box=Gene expression, Circle=Protein expression. Purple circle: increase maternal/fetal hormone ligands.

930 **Table 1.** Forward and reverse primer sequences (5'→3') of genes expression

Gene ID	Forward Primer	Reverse Primer	Accession #
ESR1	ACTGTGCAGTGTGAATGAC	TATAAAACCAAGCCTCACCT	AY033393.1
ESR2	GATGTGGGTACCGCCTTGTGC	GGCCAACTTGGTCAGGGACA	AF110817
AR	GCCCCTGACCTGGTTTTCA	TTCGGACACACTGGCTGTACA	KF227907
α MHC	ACATTGCTCACTACAGCCTTGTG	GGTCCAAGGAGAGAAACATGCT	NM_001123398
β MHC	TCTCCTCCCAGCAGCAATCC	GGTCACTGCTCCATCGTTGC	NP_001116874
ANP	GCTCCTAACGGGCAATTTGTT	CCTCCACTCCAGCCTGATGA	AF037465
BNP	GACCCAGAAGTGGCTCTAATGG	CGCATTCCCACTGCA	AF037466
IR	GACGCAGGCCGGAGATGACCA	GCTCCTGCCCCGAAGACCGACTC	XM_590552.4
IRS-1	CGCTCCAGCGAGGATCTAAG	AGGTCCTCTGGCTGCTTCTG	XM_004004977
IRS-2	CCCGAGAAGGTGGCCCGCATCA	AGCAACACGCCCGAGTCCATC	NM_003749.2
PI3K	TTGTCCAGCCACCATGATGT	TGAGCAAGAGGCTTTGGAATATT	M93252.1
AKT	CGGCTCCCTCTCCTGTTAGG	GGGATTTTCCAGCCAAGAGTACT	AF207873
mTORC1	ATCACCCCTTGCTCTCCGAACCTCTC	CCAGCTCCCGGATCTCAAACACCT	NM_001145455
Glut-4	GCTTGGCTTCTTCATCTTCACCTT	TGCTCAGACCACCCTTCCCTCCAG	NM_174604.1
IGF1	TTGGTGGATGCTCTCCAGTTC	AGCAGCACTCATCCACGATTC	DQ152962
IGF1R	TTAAAATGGCCAGAACCTGAG	ATTATAACCAAGCCTCCCAC	NM_001244612

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942 **Table 2.** List of antibodies used for Western blot

Peptide/protein target	Name of Antibody	catalog #	Species raised in; monoclonal or polyclonal	Dilution used
mTOR	mTOR (7C10) Antibody	2983	Rabbit, Polyclonal	1:500
GSK-3 β	GSK-3 β (27C10) Antibody	9315	Rabbit, Polyclonal	1:500
P-mTOR (Ser2448)	Phospho-mTOR (Ser2448) Antibody	2971	Rabbit, Polyclonal	1:500
P-GSK-3 β (Ser9)	Phospho- GSK-3 β (Ser9) Antibody	9323	Rabbit, Polyclonal	1:500
GLUT 4	Anti- GLUT4 Antibody	Ab33780	Rabbit, Polyclonal	1:500
GAPDH	Anti-GAPDH (14C10) Antibody	2118	Rabbit, Monoclonal	1:1000

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Supplementary Files

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