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Research Article

Hyperandrogenism diminishes maternal–fetal fatty acid transport by increasing FABP4-mediated placental lipid accumulation†

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Abstract

Long-chain polyunsaturated fatty acids (LCPUFAs) are critical for fetal brain development. Infants born to preeclamptic mothers or those born growth restricted due to placental insufficiency have reduced LCPUFA and are at higher risk for developing neurodevelopmental disorders. Since plasma levels of testosterone (T) and fatty acid-binding protein 4 (FABP4) are elevated in preeclampsia, we hypothesized that elevated T induces the expression of FABP4 in the placenta leading to compromised transplacental transport of LCPUFAs. Increased maternal T in pregnant rats significantly decreased n-3 and n-6 LCPUFA levels in maternal and fetal circulation, but increased their placental accumulation. Dietary LCPUFAs supplementation in T dams increased LCPUFA levels in the maternal circulation and further augmented placental storage, while failing to increase fetal levels. The placenta in T dams exhibited increased FABP4 mRNA and protein levels. In vitro, T dose-dependently upregulated FABP4 transcription in trophoblasts. Testosterone stimulated androgen receptor (AR) recruitment to the androgen response element and *trans*-activated FABP4 promoter activity, both of which were abolished by AR antagonist. Testosterone in pregnant rats and cultured trophoblasts significantly reduced transplacental transport of C^{14}-docosahexaenoic acid (DHA) and increased C^{14}-DHA accumulation in the placenta. Importantly, FABP4 overexpression by itself in pregnant rats and trophoblasts increased transplacental transport of C^{14}-DHA with no significant placental accumulation. Testosterone exposure, in contrast, inhibited this FABP4-mediated effect by promoting C^{14}-DHA placental accumulation.

Summary sentence In summary, our studies show that maternal hyperandrogenism increases placental FABP4 expression via transcriptional upregulation and preferentially routes LCPUFAs toward cellular storage in the placenta leading to offspring lipid deficiency.

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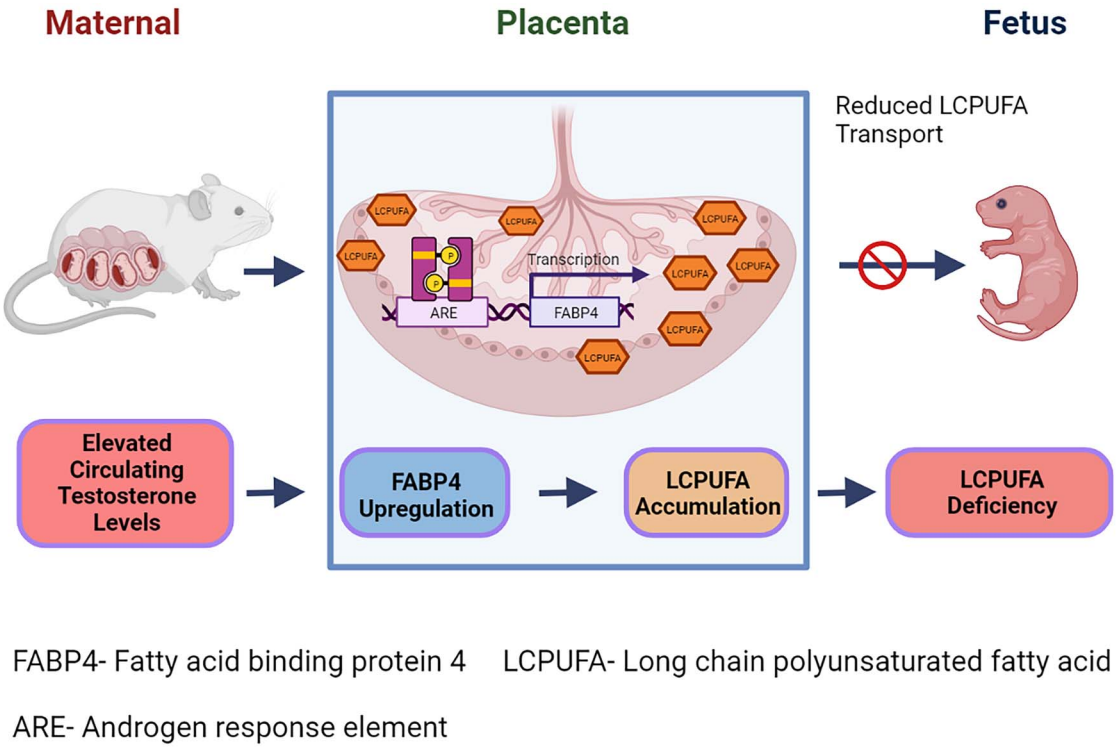
Graphical Abstract



Keywords: pregnancy, androgens, placenta, preeclampsia, fatty acids, FABP4, fetal growth

Introduction

The developing fetus requires saturated and unsaturated fatty acids as a source of energy and structural components of cell membranes. They are also precursors of bioactive molecules, including steroid hormones, prostaglandins, prostacyclins, thromboxanes, and leukotrienes, involved in brain development, blood pressure regulation, smooth muscle contraction, and blood clotting [1]. The essential long-chain polyunsaturated fatty acids (LCPUFAs) linoleic (LA; 18: 2 n-6) and alpha-linolenic (ALA; 18:3 n-3) acids and their respective long-chain derivatives, mainly arachidonic (ARA; 20:4 n-6) and docosahexaenoic (DHA; 22:6 n-3) acids, are crucial for fetal tissue growth and development [2–4]. Both the human [5] and rat fetuses [6] have limited ability to synthesize LCPUFAs, including AA and DHA [7–11]. Fetal LCPUFA demand must, therefore, be met from the maternal circulation and LCPUFA transfer across the syncytiotrophoblast, the transport epithelium of the placenta, and the primary barrier between maternal and fetal circulations [4]. The transplacental transport of LCPUFAs is first enabled by lipoprotein and endothelial lipases in the syncytiotrophoblast microvillous plasma membrane. Placental enzymatic hydrolysis of circulating maternal triglycerides and phospholipids enables the release of free fatty acids [12]. Liberated free fatty acids are then transferred from the maternal circulation across the microvillous plasma membrane by simple diffusion or via membrane-bound fatty acid transport proteins (FATPs) and fatty acid translocase/cluster of differentiation 36 (FAT/CD36). Once inside syncytiotrophoblasts, free fatty acids bind with cytosolic fatty acid-binding proteins (FABPs) and are transferred through intracellular aqueous cytoplasm to either target organelles or the fetal-facing syncytiotrophoblast basal plasma membrane for transport to the fetal circulation [13]. Long-chain polyunsaturated fatty acids are preferentially transferred across the syncytiotrophoblast, and this explains the higher content of LCPUFA, in particular of DHA, in fetal circulation compared with the maternal compartment [14, 15].

The requirement for fatty acids increases exponentially with fetal age, especially during the last weeks of intrauterine life [2, 16]. For example, human fetal brain DHA accretion occurs at a rate of ~14.5 mg/week during the last trimester [17]. In rodents, brain DHA content increases by 10-fold (from 0.64 to 6.6 mg/brain, at ~2 mg/week) during the first 3 weeks of neonatal life [18]. This sharp increase in brain DHA accretion coincides with human fetal and rodent prepubertal growth spurts when extensive axonal and dendritic outgrowth and development of synaptic connections are made [16, 19]. This exemplifies the importance of having an optimal amount of DHA in the fetal circulation during the perinatal period in both humans and rodents. Unfortunately, infants born to mothers with preeclampsia (PE) or those born growth restricted due to placental insufficiency have reduced circulatory LCPUFAs, and fat stores compared with normal babies [20–23], and are at increased risk for developing neurological [24, 25] and cardiovascular disorders [26, 27]. The reasons for the reduced transplacental transport and lower

fetal levels of LCPUFA in PE and fetal growth restriction are not clear but may be related to compromised transplacental transfer of LCPUFAs, as reflected by lower cord vein and fetal AA and DHA concentrations [21, 28, 29] accompanying increased placental accumulation [30]. Intriguingly, even maternal LCPUFA supplementation appears to be of little or no beneficial effects in mothers with PE [31–33]. Strikingly, pregnant women with PE are consistently reported to have higher circulatory and placental levels of fatty acid-binding protein 4 (FABP4) [34–43]. FABP4 is known to have a higher binding affinity to LCPUFAs [44] and regulates trophoblastic lipid transport and accumulation during placental development [45]. It has also been reported that maternal FABP4 levels are elevated in PE, even before the clinical onset of the disease [39]. However, the functional role of FABP4 in the transplacental transport of LCPUFAs and the mechanisms contributing to its placental upregulation remain unknown.

Epidemiological studies show that testosterone (T) levels are elevated 1.5- to 2.4-fold in women with PE compared to normotensive pregnant women [46] and correlate with the delivery of small-for-gestational-age babies [47]. Elevated T, as observed in polycystic ovary syndrome (PCOS), is associated with increased FABP4 mRNA in granulosa cells [48]. Experimental studies also show that elevated T increases FABP4 expression in the rat placenta [49] with an associated decrease in placental weight in rats [50], an increase in placental differentiation in sheep [51], and a decrease in placental amino acid transport capacity in rats [50]. Despite the well-documented effects of elevated maternal T on feto-placental growth, together with the available circumstantial evidence of its association with increased FABP4 expression, the direct effect of T on transplacental transport of LCPUFAs and placental FABP4 expression has not been studied. In the present study, we hypothesized that elevated T induces the expression of FABP4 in the placenta leading to compromised transplacental transport of LCPUFAs. Thus, the objectives are to determine (1) the effect of elevated T on maternal, fetal, and placental LCPUFA concentrations, (2) whether dietary LCPUFA supplementation rescues T-induced changes in fatty acid levels, (3) whether T alters placental expression of genes involved in fatty acid transport, (4) whether T regulates FABP4 transcription in the placenta, and (5) the (patho)physiological role of FABP4 on transplacental fatty acid transport.

Methods

Animals and treatment

All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison and were in accordance with those guidelines published by the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Timed pregnant Sprague–Dawley rats (gestational day [GD] 12; the presence of copulation plug is day 1), purchased from Envigo (Indianapolis, IN), were used in this study. After acclimatization, on GD 14, dams were randomly divided into control and treatment groups. Dams in the treatment group were subcutaneously injected with T propionate (0.5 mg/kg/-day, $n = 12$) for 5 days from GD 15–20. The control group received vehicle (sesame oil, $n = 12$). This dose and duration of T exposure are commonly used to mimic plasma T levels observed in PE pregnancies, and this model consistently exhibited PE features [46, 49, 52]. The rats were fed with standard rodent (Teklad global rodent diet # TD.94045, Envigo). A subset of rats in these two groups were fed an LCPUFA-enriched diet (AIN-93G, # 112325, Dyets, Inc. Bethlehem, PA) during the course of T exposure. Food and water were provided ad libitum, and all groups were housed in a room with controlled temperature and a 12-h light–dark cycle.

Serum and placenta collection

Between 9 and 10 a.m. on GD 21, rats were anesthetized using isoflurane, and maternal laparotomy was done to expose individual fetal units. Fetal blood was collected by nicking the left ventricle of the heart using 26G BD precision slide needle and 1 ml BD tuberculin slip tip syringe. Maternal blood was collected by cardiac puncture. Collected samples from six to eight fetuses of individual dams were pooled, serum separated, and processed in parallel with the corresponding maternal samples. Placentas were collected, rinsed thoroughly in ice-chilled saline, and blotted with Whatman filter paper. Maternal tissues, including liver and adipose tissue, were also collected. All samples were snap-frozen in liquid nitrogen and stored at −80 °C until further processing.

Liquid chromatography-Mass spectrometry (LC–MS/MS) quantification of LCPUFAs

Total fatty acids were extracted from maternal serum, using chloroform:methanol 2:1 (v/v) containing butylated hydroxytoluene (BHT) to prevent oxidation during sample preparation [53]. Five microliters of plasma was mixed with 1.5 ml of chloroform:methanol (2: 1 v/v). To this mixture, 300 μl of water was added to facilitate phase separation. The bottom phase was removed and mixed with 500 μl of chloroform:methanol:water (3/48/47; v/v/v), vortexed, and centrifuged for 5 min at 16 000*g* at 4 °C. The lower phase containing lipids was dried down in a speed vac and reconstituted with 200 μl of methanol:dichloromethane (1:1 v/v) containing 2 mM ammonium acetate prior to mass spectrometric analysis. For placental tissues, fatty acid extraction was performed according to the modification of the method of Brown et al. [30]. In brief, 40 mg of frozen placental tissue was homogenized in 400 μl methanol containing 50 μg/ml of BHT using a bead homogenizer. The homogenate was vortexed for 2 min and left on ice for 2 min (repeated three times). Insoluble material was cleared by centrifugation for 5 min at 16 000*g* at 4 °C. The supernatant (25 μl) was added to 250 μl MTBE and vortexed overnight at 4 °C. Ammonium acetate (150 μl of 100 mM) was added to induce phase separation. The upper organic layer was removed to a new vial, dried in a speed vac, resuspended in 125 μl of methanol:dichloromethane (1:1 v/v) containing 2 mM ammonium acetate prior to mass spectrometric analysis. All samples subjected to ESI-MS were at a concentration below 20 μM; conditions at which ion-suppression effects are minimal [54].

Mass spectra were acquired using an ABSciex TripleTOF 5600 (Sciex, Foster City, CA) equipped with an electrospray interface with a 25 μm iD capillary and coupled to an Eksigent μUHPLC (Eksigent, Redwood City, CA) according to the methods of Tran et al. [55]. An Analyst TF 1.7 software was utilized to cycle all experiments and for data processing and acquisition. The sample was analyzed in MS/MS all acquisition mode by direct infusion, in both positive and negative ionization mode. Methanol:dichloromethane (1:1 v/v) containing 2 mM ammonium acetate was used as mobile

phase. Data were analyzed with LipidView (ABSciex) software version 1.2, including smoothing, identification, removal of isotope contribution from lower mass species, and correction for isotope distribution.

Quantitative real-time polymerase chain reaction for expression of genes related to fatty acid synthesis and transport

Total RNA was extracted using the RNeasy mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RNA concentration and integrity were determined using a DS-11 spectrophotometer (DeNovix, Wilmington, DE). One microgram of total RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). After dilution, cDNA corresponding to 100 ng of RNA was amplified by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) using FAM (Invitrogen; Thermo Scientific, Grand Island, NY) as the fluorophore in a CFX96 real-time thermal cycler (Bio-Rad). Polymerase chain reaction conditions for TaqMan Gene Expression Assay were 2 min at 50 °C and 10 min at 95 °C for 1 cycle, then 15 s at 95 °C and 1 min at 60 °C for 50 cycles. Results were calculated using the $2^{-\Delta\Delta CT}$ method and expressed as fold change of the gene of interest in treated vs control (vehicle) samples. All reactions were performed in duplicate, and β-actin was used as an internal control. All gene-specific primers used in this study are provided in Supplementary Table S1.

Western blotting

Tissues were homogenized in ice-cold RIPA buffer (Cell Signaling Technology, Danver, MA) containing a protease inhibitor tablet and phosphatase inhibitor cocktail-2 and -3 (Sigma-Aldrich, St. Louis, MO). Tissue lysates were centrifuged (14 000*g* for 10 min at 4 °C), and the protein content was measured using the BCA protein assay kit (Pierce; Thermo Scientific). The supernatant was resuspended in NuPAGE lithium dodecyl sulfate sample buffer and reducing agent (Invitrogen; Thermo Scientific). Proteins (30 μg) alongside Precision Plus Standard (Kaleidoscope; Bio-Rad, Hercules, CA), and negative controls were resolved on 4–12% gradient NuPAGE Bis-Tris gels (Invitrogen) at 100 V for 2 h at room temperature and then transferred onto Immobilon-P membranes (Millipore, Billerica, MA) at 100 V for 1 h. The membranes were blocked with 5% nonfat dry milk for 1 h and then incubated overnight at 4 °C with respective primary antibodies (Human anti-FABP4, # HPA002188, Sigma-Aldrich; Rat anti-FABP4, # PA5-79231, Invitrogen; Thermo Scientific) and β-actin (#4070, Cell Signaling Technologies). After washing, the membranes were incubated with secondary antibodies (anti-rabbit conjugated with horseradish peroxidase) and detected with the Pierce ECL detection kits (Thermo Scientific, Waltham, MA). The densitometric measurement was done using ImageJ software. Results were normalized to vehicle results and expressed as ratios of the intensity of a specific band to that of β-actin.

Cell culture and treatments

BeWo cells (CCL-98; American Type Culture Collection, Manassas, VA) were cultured at 37 °C in a 5% CO_2 atmosphere in Ham F12 K medium (Wako) with 10% FBS (Hyclone), as described previously [56]. HepG2 cells (American Type Culture Collection) were maintained in Dulbecco modification of Eagle medium (DMEM; Cellgro, Manassas, VA) containing 1% L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin in 5% CO_2 at 37 °C. 3T3-L1 cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum in 5% CO_2. BeWo, HepG2, and 3T3-L1 were treated with respective medium containing increasing concentrations of T (0.1–100 nM, Sigma) for 24 h, in the absence or presence of androgen receptor (AR) antagonist (hydroxyflutamide; 100 nM).

In silico analysis of *FABP4* promoter

Androgen receptor binding sites on the human *FABP4* gene (NCBI ID:2167) promoter were identified by LASAGNA motif search tool [57]. Briefly, the analysis was performed on the entire upstream (2 kb) sequences from the transcriptional start site (TSS) using human reference core matrices (MA0007.2) from JASPAR transcription factor database [58] with the cutoff *P*-value of 0.01. The identified AR binding sites were utilized for the downstream chromatin immunoprecipitation (ChIP) assay to assess the binding efficiency of the AR to the respective motifs, followed by the luciferase assay to determine whether AR binding to these motifs positively activates the FABP4 transcriptional machinery.

ChIP and quantitative PCR

Chromatin immunoprecipitation assay was performed using a simple ChIP kit (#9005; Cell Signaling Technologies) to validate androgen response element (ARE) binding sites on human *FABP4* gene promoter as we described previously [59]. Briefly, BeWo cells were washed and cross-linked with formaldehyde treatment. Cells/tissues were lysed, and chromatin was fragmented by enzymatic digestion using micrococcal nuclease for 20 min at 37 °C with frequent mixing. The nuclei were separated, sonicated for 20 s twice with 40% power to break the nuclear membrane and clarified by centrifugation at 10 000 rpm for 10 min at 4 °C. DNA samples from the digested chromatin were separated using spin columns, and the quality was analyzed by agarose gel electrophoresis. Chromatin (5 μg of DNA in 500 μl volume for each) was then subjected to immunoprecipitation with an AR antibody (25 μl per reaction) along with positive (10 μl anti-histone antibody; Cell Signaling Technologies #4620) and negative (1 μl of rabbit IgG, Cell Signaling Technologies #2727) controls. ChIP-grade Protein G magnetic beads were used to capture the antigen–antibody complex along with specific DNA target sites. Cross-linking was then reversed by 5 mM NaCl and followed by proteinase K treatment to remove the protein contamination. Sample DNA fragments containing specific AR binding sites were purified using spin columns, quantified, and stored for further qPCR analysis. Using custom-designed primers (Supplementary Table S2) that flank the predicted putative AREs, qPCR was performed to identify the ligand-dependent AR binding to AREs in *FABP4* gene promoter, and the results were analyzed as we described previously [59].

FABP4 promoter constructs and reporter assay

Promoter sequences corresponding to the human FABP4 transcript ENST00000256104.4 were obtained from Eukaryotic Promoter Database [60]. The ChIP detected AR binding motifs located within 1 kb (AREs 1, 2, and 3) FABP4 promoter fragments containing Mlu I and Xho I flanks were custom synthesized through IDT and cloned into respective multiple cloning sites of pGL3 basic backbone,

as described previously [61]. For the reporter assay, these individual promoter constructs were transiently transfected into $\sim 6 \times 10^8$ BeWo cells using Fugene HD transfection reagent (Promega, Madison, WI) along with pmaxGFP vector to monitor the transfection efficiency. After 24 h, cells were washed and incubated in the presence of vehicle (ethanol), T (10 nM), T+ hydroxyflutamide (100 nM), and hydroxyflutamide alone for 24 h at 37 °C with 5% CO_2 in F-12K media containing 10% FBS and 1% antibiotics (Invitrogen; Thermo Scientific). Then, the cells were washed with ice-cold PBS twice and were lysed by adding 250 μl of passive lysis buffer per well (Promega). A luciferase assay was performed using the Luciferase reporter assay system (E1500; Promega), following the manufacturer's instructions. Cell lysates (20 μl) were transferred onto a 96-well plate, and GFP fluorescence was measured using FLUOstar Omega (BMG Labtech, Ortenberg, Germany). After that, 100 μl of luciferase assay reagent was dispensed in the same plate, and luminescence was measured using Fluorstar Omega. The luciferase activity was normalized with GFP fluorescence, and the results were expressed as relative luminescence.

Lentiviral particles and FABP4 overexpression

A Lenti ORF clone of human FABP4 (PS100092, OriGene, Rockville, MD) was obtained, and the lentivirus was produced by using a Lenti-vpak Packaging Kit (TR30037, OriGene) following the manufacturer's instructions. Briefly, 5×10^6 of 293T cells were plated in a 15-cm tissue culture dish and incubated for 1 day. For transfection, 5 μg lentiviral vector containing the cytomegalovirus promoter-driven FABP4 ORF and 6 μg of lentiviral packaging plasmids were mixed with 1.5 ml Opti-MEM and 33 μl turbofectin transfection reagent. After 15 min incubation at room temperature, the mixture was added to T293 cells in culture. After incubation at 37 °C for 18 h, fresh culture media was added, and viral supernatant was collected every 24 h for 3 days. The cell debris from collected viral supernatant was removed by filtration through a 0.45 μm filter. Viral titer was determined using a one-wash lentivirus titer kit (TR30038, Origene). The FABP4 lentiviral particles were concentrated by using a Lenti-X Concentrator (Cat. No. 631231, Clontech, San Jose, CA). The lentiviral particles were then injected into pregnant rats and transduced into BeWo cells. Pregnant rats were injected with lentiviral FABP4 particles intravenously via the tail vein on GD 12 and 16 at a dose of 8×10^{10} viral particles/kg. The controls were injected with the same dose of viral particles lacking FABP4. For transduction in BeWo, the cells were plated at 2.5×10^5 cells per well and, after overnight incubation, transduced with lentiviral FABP4 particles at a multiplicity of infection of 100. Polybrene (5 μg/ml) (Sigma) was added to increase the infection efficiency. After overnight culture at 37 °C, the virus-containing media was replaced with fresh media. The selection was carried out for a week with 2 μg/ml puromycin (Sigma), which was changed every 2 days. The selection was terminated when the uninfected control cells died. The FABP4 expression was confirmed by Western blotting (Supplementary Figure S1).

Placental transport of ^{14}C-DHA in pregnant rats

On GD 21, rats were administered with 0.3 ml of 2.5 μmol/kg (2 μCi/kg) ^{14}C-DHA (Moravek Biochemicals, Brea, CA) via tail vein injection [62]. Freshly prepared, 1:1 molar ratio of ^{14}C-DHA diluted in saline containing BSA (pH = 6.0–6.5) was used for the injection. Maternal and fetal blood and placenta were collected as described above after 3 h of radioisotope injection; studies show that there is minimal ^{14}C-DHA back flux at this time [63]. Serum and placental samples were lysed in biosol biodegradable tissue solubilizer (National Diagnostics) for 2 h at room temperature. The bioscint liquid scintillation fluid (National Diagnostics) was used for β-counting (Tri-Carb 4910TR liquid scintillation counter, PerkinElmer). Transport data were presented as placental disintegrations per minute (dpm) per gram placenta (representing placental uptake/accumulation of isotope) and fetal dpm per milliliter fetal serum (representing the amount of isotope transported to the fetus). Transport data were expressed for the T group relative to the controls.

Transcellular ^{14}C-DHA transport using the transwell system

The transcellular fatty acid transport studies were performed as described previously [56]. Briefly, the Corning Snapwell Transwell polyester 12-well transwell plates containing permeable membranes were coated with human placental collagen (0.01% w/v). Cells were then seeded at 100 000 cells per cm^2. The cells formed a monolayer at day 3–4 post-seeding, and all experiments were carried out on day 4 after syncytialization, which was confirmed by hPL and hCG mRNA expression and secretion of hCG, as we described previously [64]. Trypan blue was added to the apical reservoir, and no color seeping through to the basal reservoir indicated cell confluency. The cells were treated with vehicle or T (0.1–100 nM) for 24 h, and then serum-free F12 medium containing 1% fatty acid-free albumin and 10 μCi of ^{14}C-DHA was replaced in the apical reservoir of the transwell. Serum-free F12 medium containing 1% fatty acid-free albumin was added to the basal reservoir of the transwell. The dose- and time-dependent changes in transcellular transport of DHA across the BeWo monolayer were measured as the rate of appearance of DHA in the lower ("fetal side") reservoir following transfer from the upper ("maternal side") reservoir. After incubation (every 4 h for up to 24 h), aliquots of cell media from the basal reservoir were collected, and transport of ^{14}C-DHA through the BeWo cell monolayer was determined by liquid scintillation counting. After the final aliquot, cells were thoroughly washed with cold PBS to remove any surface-bound FAs, then lysed and harvested in liquid scintillation fluid and prepared for scintillation counting to estimate the intracellular accumulation of ^{14}C-DHA. The results were expressed as disintegrations per minute per microgram of protein.

Statistical analysis

All data are presented as mean ± S.E.M. The normality and homogeneity of the variances were analyzed using the one-sample Kolmogorov–Smirnov test and Anderson–Darling test, respectively. One- or two-way ANOVA followed by Bonferroni post hoc test was performed on multiple observations and unpaired Student t-test for comparison of single observations between control and T propionate groups. Repeated measures ANOVA was used to compare the time-dependent effect. Data analysis was done using GraphPad Prism for Windows (GraphPad Software, San Diego, CA). Since observations in individual pups and placentas of the same litter were not independent, an average was obtained for each litter. Therefore,

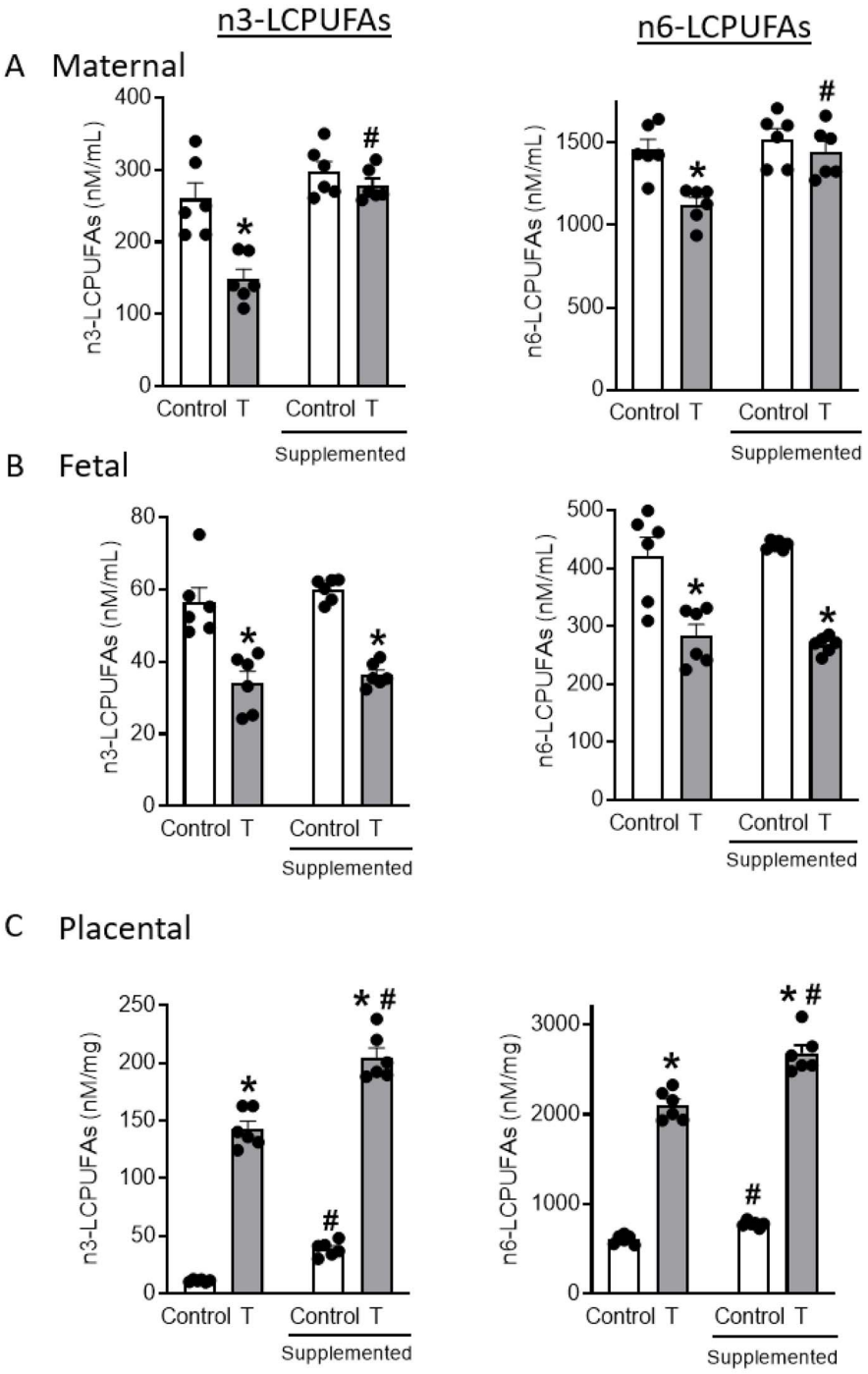


Figure 1. Absolute amounts of total *n3* and *n6* fatty acids in (A) maternal serum, (B) fetal serum, and (C) placenta of control and T dams with and without dietary LCPUFA supplementation. Fatty acids were quantified using LC–MS/MS. Data presented as mean ± SEM of six rats in each group. $^{*}P < 0.05$ vs respective control group, $^{\#}P < 0.05$ vs respective group without LCPUFA supplementation.

$n = 1$ represents averaged values in one litter. Differences were considered statistically significant at $P < 0.05$.

Results

Effect of T and dietary supplementation on placental and fetal weights

Testosterone administration during pregnancy in rats caused Intrauterine growth restriction (IUGR). At GD 21, fetal weights in T dams were diminished by 11% (control: 5.52 ± 0.21 g; T: 4.84 ± 0.27 g; $n = 6$ litters in each group; $P < 0.05$) and placental weights were reduced by 12% (control: 553 ± 14.4 mg; T: 488 ± 11.9 mg; $n = 6$ dams in each group; $P < 0.05$) in comparison to controls. Feeding a diet supplemented with LCPUFAs did not alter fetal weights in control and also did not abrogate lower fetal weight in the T group (control-supplemented: 5.65 ± 0.23; T-supplemented: 4.72 ± 0.21 g; $n = 6$ dams in each group; $P < 0.05$). Placental

Table 1. *n3*- and *n6*-LCPUFA levels in maternal serum (nM/ml)

Fatty acid	Fatty acid chain	Control	T	Control-supplemented	T-Supplemented
Omega-3 fatty acids (n-3 LCPUFAs)					
Stearidonic acid	18:4	4.99 ± 0.77	2.55 ± 0.82*	5.63 ± 0.3	2.6 ± 0.6*
Alpha-linolenic acid	18:3	180.77 ± 23.35	98.16 ± 23.79*	208.72 ± 11.1	190.39 ± 12.6#
Eicosapentaenoic acid	20:5	1.71 ± 0.25	1.05 ± 0.27*	2.31 ± 0.17	2.34 ± 0.2#
Docosahexaenoic acid	22:6	60.64 ± 2.45	40.2 ± 4.39*	66.42 ± 8.15	62.53 ± 6.71#
Docosapentaenoic acid (n-3)	22:5	11.84 ± 1.29	6.97 ± 1.28*	13.99 ± 0.72	10.79 ± 1.07#
Docosatrienoic acid	22:4	0.35 ± 0.05	0.21 ± 0.06*	0.37 ± 0.04	0.33 ± 0.04#
Total n-3 LCPUFAs		260.29 ± 26.37	149.14 ± 13.61*	297.45 ± 14.4	278.99 ± 9.14#
Omega-6 fatty acids (n-6 LCPUFAs)					
Gamma-linolenic acid	18:3	296.26 ± 36.2	166.85 ± 35.82*	284.72 ± 24.94	279.89 ± 26.81#
Linoleic acid	18:2	1060.94 ± 24.26	903.73 ± 35.64*	1124.99 ± 110.72	1068.03 ± 130.34#
Arachidonic acid	20:4	55.37 ± 3.63	36.75 ± 4.69*	60.46 ± 3.8b	53.33 ± 3.42#
Dihomo-gamma-linolenic acid	20:3	2.65 ± 0.38	1.45 ± 0.4*	2.73 ± 0.23	2.45 ± 0.23#
Eicosadienoic acid	20:2	4.48 ± 0.51	2.51 ± 0.59*	4.11 ± 0.38	3.3 ± 0.34#
Docosapentaenoic acid (n-6)	22:5	30.05 ± 1.87	8.37 ± 4.23*	37.17 ± 2b	27.99 ± 2.65*#
Docosatetraenoic acid	22:4	8.19 ± 0.98	3.26 ± 1.29*	7.82 ± 0.24	7.62 ± 0.21#
Docosadienoic acid	22:2	0.09 ± 0.02	0.05 ± 0.02*	0.09 ± 0.02	0.08 ± 0.02#
Total Omega 6 fatty acids		1458.04 ± 62	1122.99 ± 44.42*	1522.1 ± 101.94	1442.68 ± 63.99#

Values are expressed as mean ± SEM. $n = 6$ in each group. *$P < 0.05$ vs respective control group, #$P < 0.05$ vs respective group without supplementation.

efficiency (fetal to placental weight ratio) in T-treated animals was not statistically different from controls. LCPUFA supplementation did not alter placental weight in control, however, did abrogate lower placental weight in the T group (control-supplemented: 584 ± 19 mg; T-supplemented: 557 ± 21 mg; $n = 6$ dams in each group). Placental efficiency was significantly lower in the T-supplemented group compared with the control-supplemented group. No significant differences were noted in daily feed intake and mean litter size between control and T dams with or without LCPUFA supplementation. Overall, elevated maternal T caused IUGR, and LCPUFA supplementation restored placental, but not fetal, weights in T dams.

Effect of T and dietary supplementation on LCPUFA concentrations in maternal and fetal circulations

Elevated T significantly decreased total n-3 and n-6 fatty acids by 43 and 23% in maternal (Figure 1A), and 40% and 33% in fetal (Figure 1B) circulations, respectively, compared with controls ($n = 6$ dams; $P < 0.05$). Notably, maternal and fetal concentrations of ALA (maternal: ↓46%; fetal: ↓40%), DHA (maternal: ↓34%; fetal: ↓40%), linoleic acid; LNA (maternal: ↓15%; fetal: ↓32%), and ARA (maternal: ↓34%; fetal: ↓27%) were significantly decreased in the T group compared with controls (Tables 1 and 2).

Dietary LCPUFA supplementation negated the marked decrease observed in total fatty acids in the maternal circulation of T dams by increasing n-3 by 67% and n-6 by 20%, but was without significant effect in the controls (Figure 1A; $n = 6$ dams; $P < 0.05$). Long-chain polyunsaturated fatty acid supplementation, therefore, normalized total n-3 and n-6 fatty acid concentrations in the maternal circulation of T dams by restoring levels of ALA, DHA, LNA, and ARA to those found in control-supplemented dams (Tables 1 and 2).

When compared to the respective counterparts on a standard diet, dietary LCPUFA supplementation in T dams failed to abrogate deficiencies in total n-3 and n-6 fatty acids in the T fetal circulation (n-3: ↓40%; n-6: ↓39%) and was without significant effects in control fetuses (Figure 1B; $n = 6$ dams; $P < 0.05$). Fetuses in the LUPUFA-supplemented T group thus maintained lower n-3 and n-6 fatty acids by 40 and 39%, respectively, as well diminished fetal concentrations of ALA (↓45%), DHA (↓36%), LNA (↓36%), and ARA (↓47%) compared with control-supplemented fetuses (Tables 1 and 2).

Effect of T and dietary supplementation on LCPUFA concentrations in the placenta

Elevated T significantly increased total n-3 and n-6 fatty acids in the placentae of T dams compared to controls (n-3: ↑1166%; n-6: ↑245%; Figure 1C; $n = 6$ dams; $P < 0.05$). Specifically, placental concentrations of ALA (↑252%), DHA (↑1218%), LNA (↑89%), and ARA (↑218%) were increased in the placentae of T dams (Table 3).

Dietary LCPUFA supplementation augmented total n-3 and n-6 fatty acid levels in the placentae of T dams (n-3: ↑43%; n-6: ↑27%) as well as control dams (n-3: ↑245%; n-6: ↑28%) compared with their respective counterparts on the standard diet. Thus, placental n-3 and n-6 fatty acids were significantly higher in T-supplemented dams (n-3: ↑426%; n-6: ↑244%) compared with control-supplemented dams. Notably, increased placental levels of ALA (↑330%), DHA (↑518%), LNA (↑214%), and ARA (↑161%) were observed in the placenta of T-supplemented dams compared with control-supplemented dams (Table 3). Total fatty acid levels in fetal liver and adipose tissue, nevertheless, were comparable in control and T dams (Supplementary Figure S2).

Effect of T on the expression of fatty acid synthesis and transport genes in the placenta

To determine whether fetal deficiency and placental accumulation of LCPUFAs in T dams correlated with alteration fatty acid synthesis/transport in the placenta, placental mRNA levels of genes involved in the fatty acid synthesis, FATP, and FABP were determined with qRT-PCR. As shown in Figure 2A and B, while there were no significant changes in mRNA expression placental fatty acid synthesis genes, FATPs and translocase (CD36), lipoprotein lipase mRNA expression was increased by 2-fold in T placentae compared with controls

Table 2. *n3*- and *n6*-LCPUFA levels in fetal serum (nM/ml)

Fatty acid	Fatty acid chain	Control	T	Control-supplemented	T-Supplemented
Omega-3 fatty acids (n-3 LCPUFAs)					
Stearidonic acid	18:4	1.44 ± 0.20	1.04 ± 0.18*	1.54 ± 0.06	1.09 ± 0.22*
Alpha-linolenic acid	18:3	22.92 ± 2.37	13.57 ± 3.46*	24.42 ± 0.03	13.56 ± 1.07*
Eicosapentaenoic acid	20:5	0.53 ± 0.04	0.36 ± 0.06*	0.69 ± 0.14	0.23 ± 0.07*
Docosahexaenoic acid	22:6	30.04 ± 1.55	17.94 ± 3.74*	31.61 ± 0.83	20.09 ± 0.49*
Docosapentaenoic acid (n-3)	22:5	1.39 ± 0.14	1.20 ± 0.09	1.64 ± 0.03	1.54 ± 0.17
Docosatrienoic acid	22:4	0.15 ± 0.01	0.07 ± 0.03*	0.15 ± 0.01	0.05 ± 0.01*
Total n-3 LCPUFAs		56.47 ± 4.08	34.17 ± 3.15*	60.04 ± 1.27	36.34 ± 0.81*
Omega-6 fatty acids (n-6 LCPUFAs)					
Gamma-linolenic acid	18:3	39.87 ± 3.89	23.43 ± 5.87*	41.32 ± 1.65	19.74 ± 2.09*
Linoleic acid	18:2	350.25 ± 25.73	239.75 ± 42.05*	360.28 ± 4.58	230.72 ± 4.36*
Arachidonic acid	20:4	19.70 ± 1.48	14.46 ± 2.34*	23.15 ± 0.47	12.39 ± 2.07*
Dihomo-gamma-linolenic acid	20:3	1.06 ± 0.07	0.53 ± 0.16*	1.61 ± 0.13#	0.55 ± 0.11*
Eicosadienoic acid	20:2	1.10 ± 0.09	0.62 ± 0.16*	1.46 ± 0.11	0.48 ± 0.10*
Docosapentaenoic acid (n-6)	22:5	8.71 ± 0.83	3.49 ± 1.49*	9.13 ± 0.29	3.76 ± 0.51*
Docosatetraenoic acid	22:4	1.15 ± 0.09	0.89 ± 0.12	2.89 ± 0.44#	0.56 ± 0.11*#
Docosadienoic acid	22:2	0.06 ± 0.01	0.04 ± 0.009*	0.36 ± 0.14	0.06 ± 0.01*
Total Omega 6 fatty acids		421.88 ± 31.78	283.21 ± 20.51*	440.21 ± 3.09	268.2 ± 5.91*

Values are expressed as mean ± SEM. $n = 6$ in each group. *$P < 0.05$ vs respective control group, #$P < 0.05$ vs respective group without supplementation.

Table 3. *n3*- and *n6*-LCPUFA levels in placenta (nM/mg)

Fatty acid	Fatty acid chain	Control	T	Control-supplemented	T-Supplemented
Omega-3 fatty acids (n-3 LCPUFAs)					
Stearidonic acid	18:4	1.14 ± 0.57	57.48 ± 4.85*	19.62 ± 4.18#	77.85 ± 7.83*#
Alpha-linolenic acid	18:3	13.25 ± 1.31	46.59 ± 6.56*	18.98 ± 4.3	81.69 ± 4.65*#
Eicosapentaenoic acid	20:5	0.4 ± 0.06	67.25 ± 5.92*	3.66 ± 0.43#	92.58 ± 3.07*#
Docosahexaenoic acid	22:6	46.81 ± 2.94	617.11 ± 35.71*	146.11 ± 8.91#	903.55 ± 31.92*#
Docosapentaenoic acid (n-3)	22:5	5.39 ± 0.39	67.98 ± 5.92*	44.34 ± 16.68#	72.23 ± 7.4*
Docosatrienoic acid	22:4	0.78 ± 0.06	1.34 ± 0.23*	0.86 ± 0.09	1.17 ± 0.17*
Total n-3 LCPUFAs		11.29 ± 0.42	142.96 ± 6.49*	38.93 ± 2.65#	204.85 ± 8.31*#
Omega-6 fatty acids (n-6 LCPUFAs)					
Gamma-linolenic acid	18:3	11.25 ± 0.82	27.15 ± 0.98*	20.38 ± 0.76#	16.25 ± 2.88#
Linoleic acid	18:2	358.44 ± 13.73	677.93 ± 11.88*	268.48 ± 10.56#	843.92 ± 50.02*#
Arachidonic acid	20:4	174.33 ± 10.26	554.62 ± 14.46*	229 ± 9.13	597.12 ± 29.44*
Dihomo-gamma-linolenic acid	20:3	6.92 ± 0.46	17.99 ± 1.19*	8.62 ± 0.67	26.83 ± 1.29*#
Eicosadienoic acid	20:2	13.37 ± 0.36	491.54 ± 41.1*	87.73 ± 4.62#	748.64 ± 53.45*#
Docosapentaenoic acid (n-6)	22:5	26.12 ± 1.31	305.21 ± 36.08*	133.36 ± 5.76#	402.11 ± 14.49*
Docosatetraenoic acid	22:4	17.36 ± 1.99	24.65 ± 1.9*	31.03 ± 2.84#	41.01 ± 2.88*#
Docosadienoic acid	22:2	1.41 ± 0.11	2.06 ± 0.26*	1.37 ± 0.12	4.69 ± 0.48*#
Total Omega 6 fatty acids		609.19 ± 20.37	2101.17 ± 67.51*	779.96 ± 13.73#	2680.59 ± 90.79*#

Values are expressed as mean ± SEM. $n = 6$ in each group. *$P < 0.05$ vs respective control group, #$P < 0.05$ vs respective group without supplementation.

($n = 6$ in each group; $P < 0.05$). Strikingly, placental FABP4 was the only FABP that significantly increased (4-fold) in the placentae of T dams compared with controls (Figure 2C; $n = 6$ in each group; $P < 0.05$). Western blotting showed that FABP4 protein levels were significantly increased by 3.3-fold in T placentae compared with controls (Figure 2D; $n = 6$ in each; $P < 0.05$). Increased FABP4 expression, however, was limited to placentae as no differences in FABP4 mRNA levels were observed in fetal liver and adipose tissue of T compared with control groups (Figure 2E; $n = 6$ in each).

Effect of T on FABP4 mRNA expression in cultured trophoblast cells in vitro

We next tested whether T directly regulates the expression of FABP4 by examining T effects in cultured human trophoblast (BeWo) cells. Testosterone, at a clinically relevant concentration range (0.1–10 nM), induced dose-dependent increases in *FABP4* mRNA expression up to 3.7-fold in the trophoblasts, and this effect was prevented by the selective AR antagonist hydroxyflutamide (100 nM) (Figure 3A; $n = 4$; $P < 0.05$). Also, T at 10 nM induced a 6-fold increase in FABP4 protein levels compared to vehicle (Figure 3B; $n = 4$; $P < 0.05$). Consistent with the effects observed in vivo, T did not affect *FABP4* mRNA expression in cultured hepatocytes (hepG2) and adipocytes (3T3-L1) (Figure 3C; $n = 4$ in each). Testosterone, therefore, selectively upregulated *FABP4* transcription in human trophoblast cells but not in hepatocytes and adipocytes.

AR binding sites on *FABP4* gene promoter

We probed *FABP4* for promoter analysis. Bioinformatic analysis of *FABP4* promoter using the LASAGNA motif search tool identified 5 putative AREs within the −2 kb upstream region from the FABP4 TSS (Figure 4A). We further probed

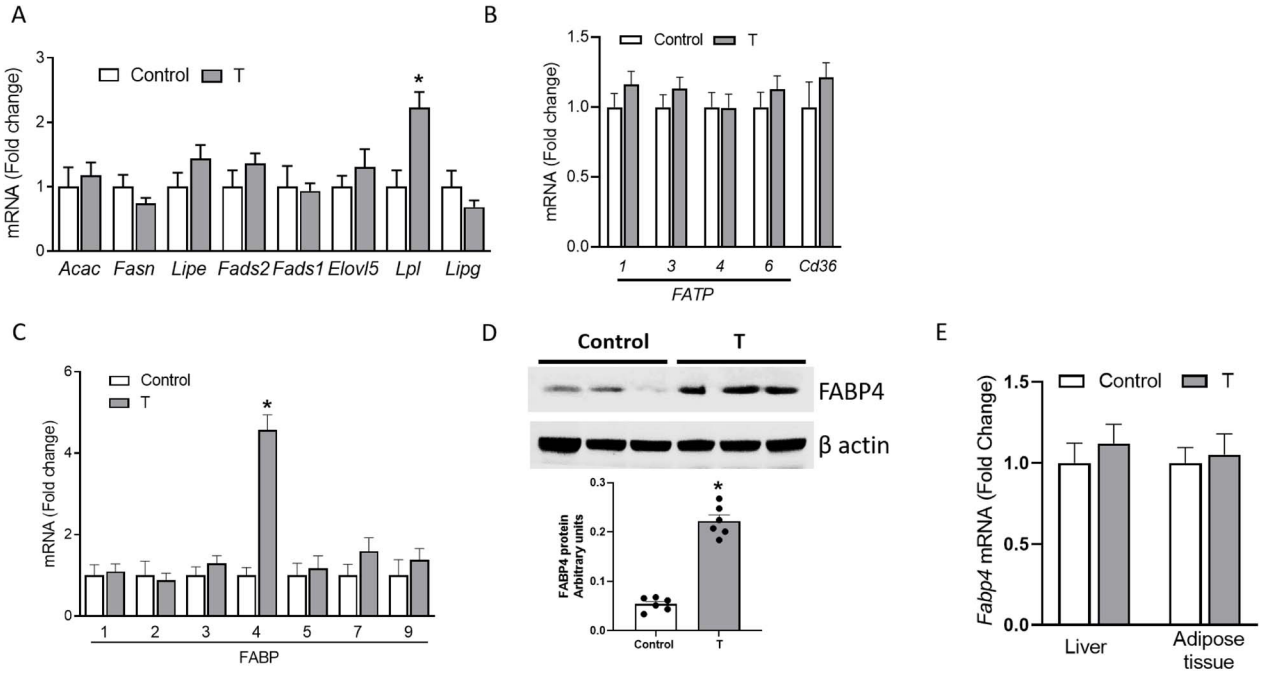


Figure 2. Effect of elevated T on mRNA expression of genes involved in (A) fatty acid synthesis, (B) FATP, and (C) FABPs in the placenta. (D) Effect of elevated T on FABP4 protein expression in the placenta. Representative Western blots for FABP4 and β-actin are shown at *top*; blot density obtained from densitometric scanning of FABP4 normalized to β-actin is shown at *bottom*. (E) FABP4 mRNA expression in the liver and adipose tissue of control and T dams. mRNA expression was normalized to β-actin. Data presented as mean ± SEM of six rats per group. $^*P < 0.05$ vs control. Acac, Acetyl-CoA Carboxylase Alpha; Fasn, Fatty Acid Synthase; Lipe, Lipase E, Hormone Sensitive Type; Fads2, Delta-6-Desaturase; Fads1, Delta-5 Desaturase; Elovl5, ELOVL Fatty Acid Elongase 5; Lpl, Lipoprotein Lipase; Lipg, Lipase G, Gastric Type.

FABP4 with ChIP assay to identify if any of these AREs interacts with AR in the presence of T in trophoblast cells. Our results show that ARE1, ARE2, and ARE3 interacted with AR in a ligand-dependent fashion showing 1.8-, 2.7-, and 2.8-fold enrichment, respectively, when treated with T compared with controls (Figure 4B; $n = 4$; $P < 0.05$). Other predicted AREs (ARE4 and ARE5) did not show significant ligand-dependent enrichment.

Functional effect of AR–ARE interaction on *FABP4* gene expression

Since AR interacted with ARE1, 2, and 3, we further probed whether the AR-ARE binding could result in the activation of *FABP4* promoter. We cloned 1 kb promoter region of *FABP4* and performed a reporter assay. Results showed that AR was able to induce transcription in a ligand-dependent manner. Testosterone increased luciferase activity by ~3-fold and was significantly higher than vehicle (Figure 4C; $n = 4$; $P < 0.05$). Hydroxyflutamide treatment abolished T-mediated luciferase activity (Figure 4C; $n = 4$; $P < 0.05$). Our data thus suggest that the functional AR binding site in the −1 kb promoter region of the *FABP4* gene has a promoter engaging function.

Effect of T and FABP4 overexpression on placental DHA transport in pregnant rats in vivo

To understand the functional effect of T and the role of FABP4 overexpression on transplacental transport LCPUFAs, we performed radiotracer transport studies using C^{14}-DHA. In normal (wild-type) pregnant rats, elevated T inhibited transplacental C^{14}-DHA transport to the fetus by 46% accompanied by a 2-fold increase in C^{14}-DHA accumulation in placentae compared to controls (Figure 5; $n = 6$; $P < 0.05$). Overexpression of FABP4 in pregnant rats augmented transplacental transport of C^{14}-DHA to the fetus by 2.2-fold without significant accumulation in placentae compared with wild-type pregnant rats (Figure 5; $n = 6$; $P < 0.05$). Testosterone treatment inhibited the FABP4 overexpression-induced increase in transport of DHA to the fetus by 66% with an accompanying 2-fold increase in C^{14}-DHA accumulation in placentae (Figure 5; $n = 6$; $P < 0.05$).

Effect of T and FABP4 overexpression on transcellular DHA transport in cultured trophoblast cells in vitro

In normal BeWo trophoblast cells, T dose- and time-dependently decreased the rate of C^{14}-DHA transfer across the monolayer (Figure 6A; $n = 4$; $P < 0.05$), and this was associated with an increase in the intracellular accumulation of C^{14}-DHA at 24 h time point (Figure 6B; $n = 4$; $P < 0.05$). FABP4 overexpression in BeWo cells induced a time-dependent increase in transcellular transport of C^{14}-DHA, and T treatment inhibited the FABP4 overexpression-induced increase in transcellular C^{14}-DHA transport (Figure 6C; $n = 4$; $P < 0.05$). FABP4 overexpression in BeWo cells did not alter intracellular C^{14}-DHA levels compared to normal trophoblasts, but T treatment induced significant accumulation of C^{14}-DHA in FABP4-overexpressed cells (Figure 6D; $n = 4$; $P < 0.05$).

Discussion

The major finding of this study is that, at clinically relevant concentrations, elevated maternal T levels during pregnancy

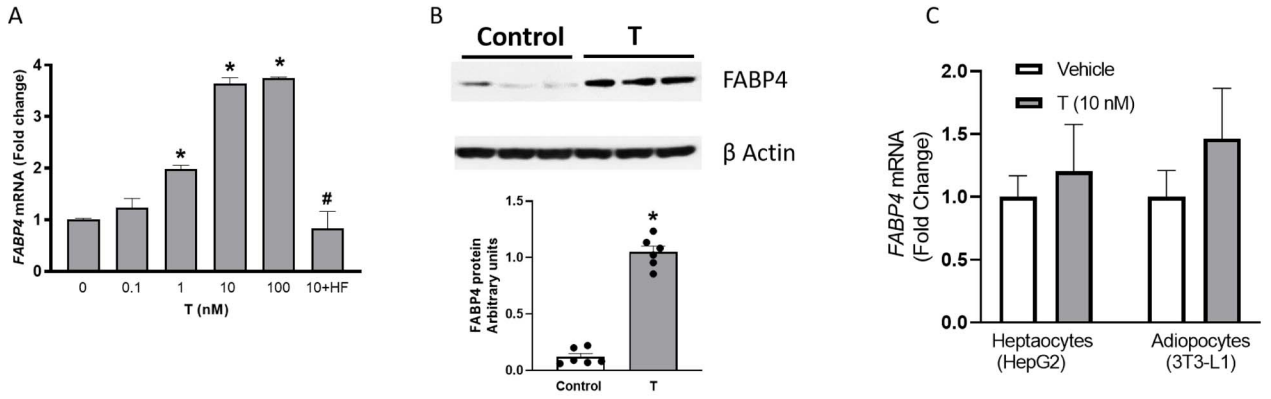


Figure 3. Effect of T on FABP4 (A) mRNA and (B) protein expression in cultured trophoblast cells (BeWo) in vitro. (C) Effect of T on *FABP4* mRNA expression in cultured hepatocytes (HepG2) and adipocytes (3T3-L1). Representative Western blots for FABP4 and β-actin are shown at *top*; blot density obtained from densitometric scanning of FABP4 normalized to β-actin is shown at *bottom*. mRNA expression was normalized with β-actin. Data presented as mean ± SEM from four independent experiments. $^{*}P < 0.05$ vs vehicle control, $^{\#}P < 0.05$ vs 10 nM T treatment. HF, hydroxyflutamide (100 nM).

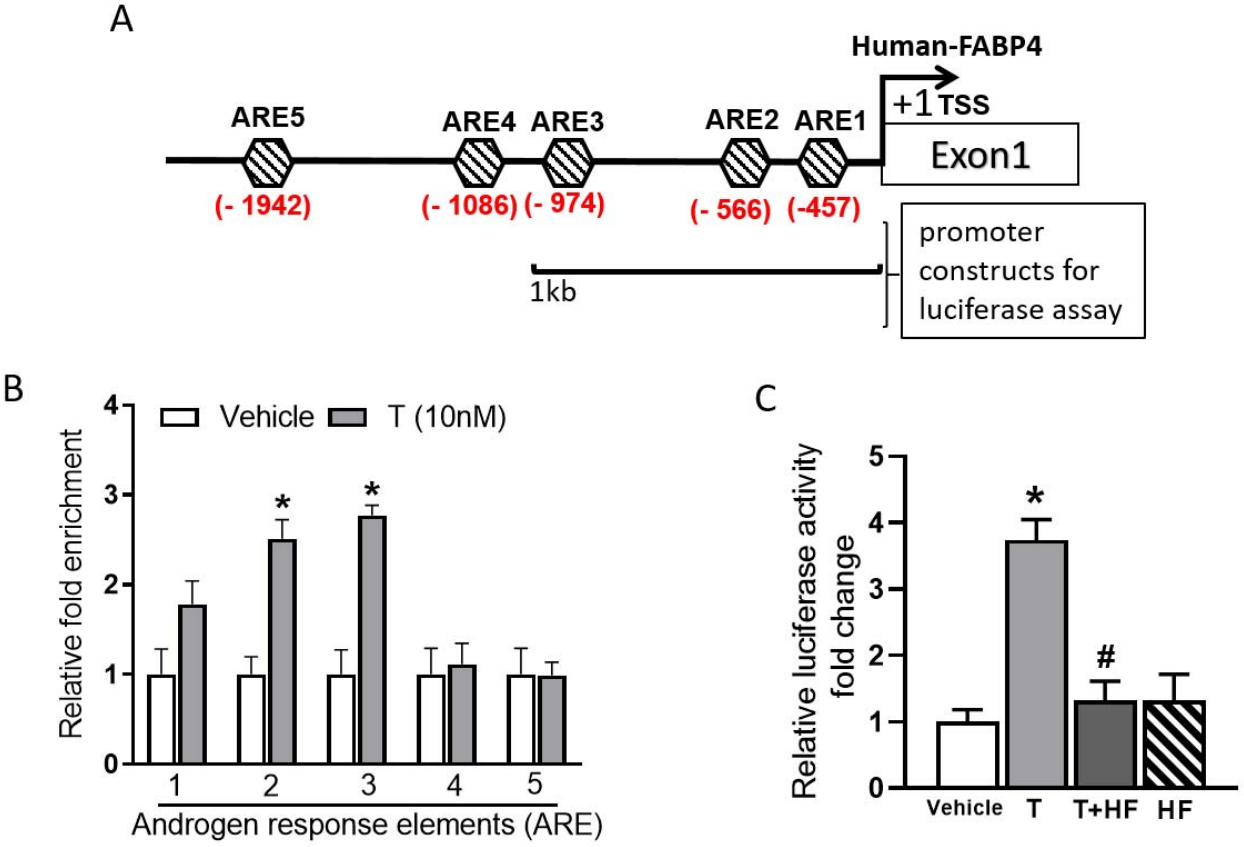


Figure 4. Bioinformatic analysis of *FABP4* gene promoter and functional validation of androgen receptor binding motifs. (A) Schematics showing the location of AREs on *FABP4* promoter, (B) ChIP assay showing T-induced AR interaction with a putative ARE (C) reporter assay showing luciferase activity in BeWo cells transfected with the reporter plasmid containing ARE 1, 2 and 3 in the presence of T (10 nM) with and without hydroxylflutamide (HF, 100 nM). Data presented as mean ± SEM from four independent experiments. $^{*}P < 0.05$ vs Vehicle, $^{\#}P < 0.05$ vs T treatment.

led to increased accumulation of LCPUFAs in the placenta with reduced concentrations in both maternal and fetal circulations. In T-treated dams, dietary LCPUFA supplementation normalized maternal LCPUFA levels and augmented placental LCPUFA accumulation, but failed to increase fetal LCPUFA levels. Testosterone directly upregulated placental *FABP4* mRNA transcripts by positively regulating *FABP4* transcription through a functional ARE in the *FABP4* gene promoter; yet, the transplacental transport of LCPUFAs was reduced. FABP4 overexpression by itself increased transplacental transport of DHA, but elevated maternal T prevented this FABP4 overexpression effect by promoting LCPUFA accumulation in the placenta. Therefore, we suggest that maternal hyperandrogenism during pregnancy induces placental FABP4

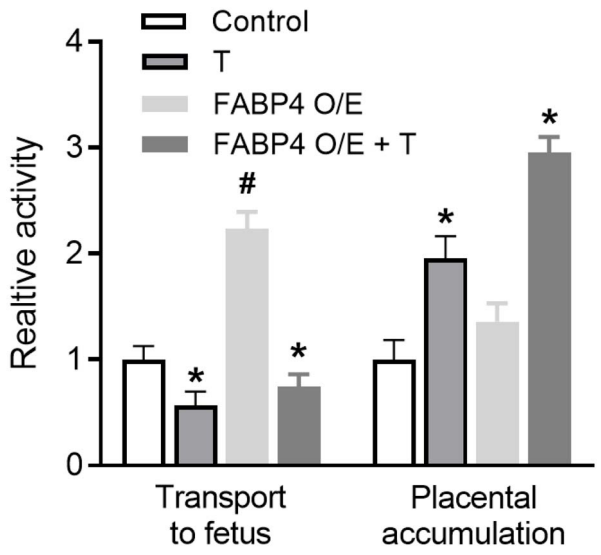


Figure 5. Effect of T and FABP4 overexpression on transplacental DHA transport in pregnant rats in vivo. Radiotracer transport studies using C^{14}-DHA were performed and placental transport to the fetus (fetal dpm per milliliter serum) and placental accumulation (placental dpm per gram placenta) were measured. Data presented as mean ± SEM from $n = 6$ dams in each group. $^{*}P < 0.05$ vs respective control, $^{\#}P < 0.05$ vs wild type. FABP O/E, FABP4 overexpressed.

expression via transcriptional upregulation and preferentially routes LCPUFAs toward placental intracellular storage leading to offspring lipid deficiency.

Although PE is one of the major causes of maternal and perinatal mortality and morbidity, the pathophysiology of this disease has yet to be completely understood. Placental AR expression is increased in women with PE, coincident with increased placental T production [65]. We have previously shown that elevated maternal T in pregnant rats, at levels similar to that in women with PE, decreased transplacental transport of amino acid, but not glucose, to the fetus coincident with decreased offspring birth weight [50]. In this study, we not only confirm that elevated maternal T decreases fetal growth, but we also provide novel findings demonstrating decreased absolute amounts of n-3 and n-6 LCPUFA in both maternal and fetal circulations, especially essential fatty acids such as ARA and DHA. The decreased maternal LCPUFA concentrations were not due to inhibition of its synthesis, as the expression of Δ5- and Δ6-desaturase in placentae, liver, and adipose tissue (Supplementary Figure S3), which are major sites of synthesis, is not altered in T dams. Our findings that T did not alter regulatory enzymes of fatty acid synthesis are not consistent with the reports of Kelly et al. [66], which showed that T suppressed fatty acid synthesis. This discrepancy could be related to the T status and sex differences. Kelly et al. [66] examined the effects of T replacement in orchiectomized males, while our study examined the impact of elevated T in pregnant females. The increased uptake and storage of LCPUFAs in the placentae of T dams in this study suggests that such ectopic lipid accumulation may contribute to decreased LCPUFA concentrations in both maternal and fetal circulations, similar to deficiencies that reported for women with PE [21]. Whether T decreases intestinal absorption of fatty acids, however, needs to be examined in the future.

Increased placental storage of fatty acids along with low fetal circulatory LCPUFA concentrations suggests that the transfer of fatty acids across the placenta may be impaired in T rats. This idea is supported by dietary supplementation of LCPUFA enhancing maternal and placental concentrations of LCPUFAs, while failing to abrogate fetal LCPUFA deficiencies in T rats. This premise is further strengthened by our radioactive tracer experiment that shows elevated T increasing placental accumulation of DHA, while at the same time decreasing transplacental transport of DHA to the fetus. Our in vitro studies of T dose-dependently increasing intracellular DHA accumulation in parallel with decreased transcellular DHA transport in BeWo cells further confirm that T stimulates increased storage of fatty acids in placental trophoblast cells while decreasing their transport to the fetus. Although previous studies have shown that lipid storage occurs in placentae of the rat L-NAME model of PE [67], reminiscent of lipid accumulation in human PE placentae [21], we provide the first evidence that maternal hyperandrogenism acting via placental AR stimulates ectopic storage of LCPUFAs in the placenta while limiting the transfer of these critical nutrients to the fetus.

The transport of fatty acids through the placenta is orchestrated by several proteins, including lipoprotein lipase, fatty acid transfer proteins such as FATP-1-6, CD36, and cytoplasmic FABP-1-9 [13]. Placentae from our T groups, however, demonstrated increased expression of lipoprotein lipase and FABP4. Lipoprotein lipase is the key enzyme abundantly expressed on the maternal side of the placenta [12] involved in hydrolyzing circulating triglycerides to increase the availability of "free" fatty acids for uptake into syncytiotrophoblasts. Increased expression of lipoprotein lipase in T placenta could, therefore, be responsible for increased uptake of fatty acids into the placenta, similar to its role in promoting fatty liver [68]. The mechanism leading to increased expression of lipoprotein lipase in the T placenta, however, needs to be clarified.

Once inside the syncytiotrophoblast, fatty acids bind to FABPs and are shuttled from the apical (maternal facing) to the basolateral (fetal facing) plasma membrane for delivery to the fetus. There are nine family members of FABPs, but our studies only demonstrated the expression of FABP-1, -2, -3, -4, -5, -7, and -9 in rat placentae. Out of these FABPs expressed, only FABP4 expression was increased in T placentae. Importantly, this is also the only FABP4 increased in the placentae of women with PE [34–43]. Placental upregulation of FABP4, however, is not a compensatory response to T-induced decreases in fetal LCPUFA concentrations, because T-exposed cultured trophoblasts upregulate *FABP4* mRNA, suggesting that FABP4 is a physiological target for T and that androgens normally upregulate placental FABP4 expression. Testosterone induction of FABP4 expression was indeed placenta-specific since T was consistently unable to stimulate FABP4 expression in hepatocyte and adipocyte cultures. Using the AR antagonist hydroxyflutamide, we demonstrated AR-mediated T stimulation of FABP4 in trophoblast. This effect is expected as the trophoblast cells express AR, consistent with previous human studies [65, 69]. In silico analysis of the entire upstream sequences of the *FABP4* transcription start codon revealed multiple AREs within human *FABP4* promoter, which can bind to AR. ChIP-PCR analysis using specific AR antibodies showed that AR was recruited to AREs in the human *FABP4* promoter in trophoblast cells.

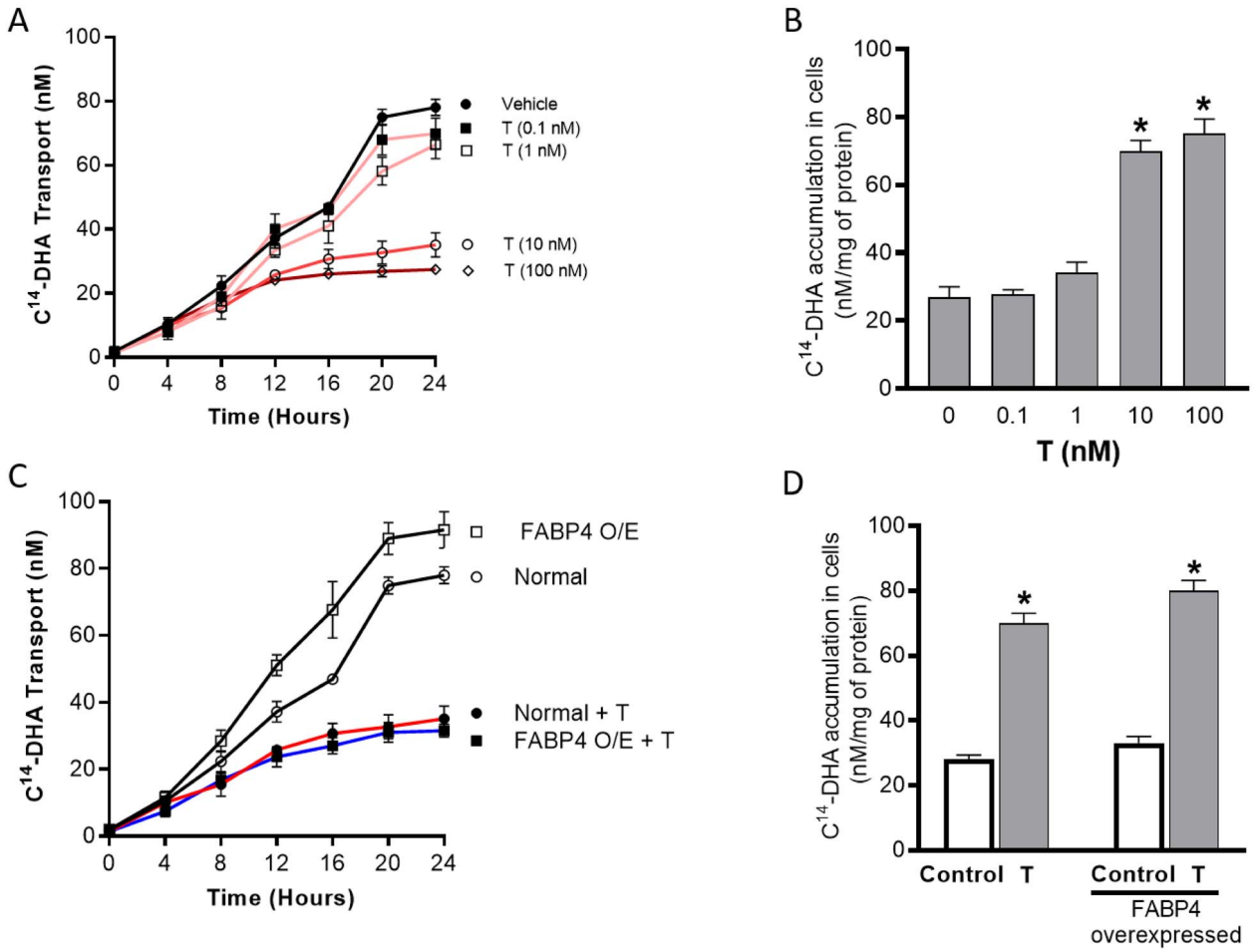


Figure 6. Effect of T and FABP4 overexpression on transcellular DHA transport in cultured BeWo cells in vitro. (A) T-induced dose- and time-dependent changes in transcellular C^{14}-DHA transport from apical to the basal reservoir in normal trophoblasts, (B) dose-dependent changes in intracellular C^{14}-DHA accumulation in normal trophoblasts after 24 h of T treatment, (C) Time-dependent changes in transcellular C^{14}-DHA transport in normal and FABP4-overexpressed trophoblasts in the presence and absence of T (10 nM) treatment. (D) Changes in intracellular C^{14}-DHA accumulation in normal and FABP4-overexpressed trophoblasts in the presence and absence of 24 h T (10 nM) treatment. Data presented as mean ± SEM of four independent experiments. $^{*}P < 0.05$ vs control. FABP O/E, FABP4 overexpressed.

These data provide the first line of evidence regarding the role of AR in mediating androgen stimulation of FABP4 expression in trophoblast cells. Indeed, this premise is supported by AR-ARE3-mediated, T-induced *trans*-activation of human *FABP4* promoter-driven luciferase reporter gene in trophoblast cells in vitro. Since AR interactions with co-activators or co-repressors can regulate ARE-regulated genes [69], future studies should discern whether co-regulators additionally contribute to T-induced trophoblast-specific augmentation of FABP4 expression.

Although FABP4 is shown to have a higher binding affinity to LCPUFAs [70], there is no direct evidence regarding the function of FABP4 in placental fatty acid transport. This study, however, is the first, to our knowledge, showing that FABP4 is involved in transplacental DHA transport. Overexpression of FABP4 in pregnant rats and BeWo trophoblast cells increased transplacental transport of DHA with no significant accumulation of DHA in the placenta. In contrast, the presence of T dramatically decreased the FABP4 overexpression-induced increase in DHA transport in pregnant rats with a simultaneous increase in placental DHA accumulation. The in vitro studies in BeWo cells thus confirm that T directly promotes disruption of cellular DHA trafficking. It is intriguing, therefore, as to the mechanism by which upregulated placental FABP4 fails to adequately maintain the transfer of fatty acids across the placenta in T rats and women with PE. Mass spectrometry-based screens have independently identified numerous posttranslational modifications on FABP4, including phosphorylation, acetylation, and carbonylation [71–73], but only phosphorylation has been validated in in vitro systems, and the functional relevance of such modification(s) remains to be addressed [73]. It thus remains to be elucidated whether posttranslational modifications contribute to FABP4 dysfunction in the placenta of T rats and women with PE. Although we focused on DHA transport in this study, it would be interstring to confirm if similar regulatory effects are operational in the transplacental transport of other LCPUFAs. Further, it is also necessary to examine if T and FABP4 effects on placental fatty acid handling vary depending on fetal sex.

In conclusion, this study indicates that while elevated maternal T increases placental FABP4 expression by an AR-mediated mechanism, it concomitantly impair transport of fatty acids across the placenta leading to offspring lipid

deficiency. These results suggest a role for AR-mediated maternal T action as a possible dysregulator of placental lipid trafficking. It is, therefore, interesting to speculate that in human pregnancies with PE, or in women with hyperandrogenic PCOS in whom the incidence of PE is increased [74], ectopic lipid storage in the placenta decreased maternal and fetal LCPUFA levels and inconsistent beneficial effects of DHA supplementation during PE may all indeed be AR-mediated. In a nonhuman primate model for PCOS, increasing maternal T during early-to-mid gestation diminished fetal circulating levels of total free fatty acids later in gestation [74]. The ability of elevated maternal T to influence the transfer of critical nutrients to the fetus may contribute to some of the adverse effects of T on fetal growth and development observed in both women with PE and with PCOS. Strategies that target excessive AR-mediated dysregulation of LCPUFA trafficking in the placenta could lead to new approaches in preventing the dyslipidemic effects in pregnancies complicated by PE and fetal growth restriction.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of interest

The authors have no conflicts of interest to declare.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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