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Research Article

Dietary Progesterone Contributes to Intratissue Levels of Progesterone in Male Mice

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Abstract

Progesterone serum levels have been identified as a potential predictor for treatment effect in men with advanced prostate cancer, which is an androgen-driven disease. Although progesterone is the most abundant sex steroid in orchiectomized (ORX) male mice, the origins of progesterone in males are unclear.

To determine the origins of progesterone and androgens, we first determined the effect of ORX, adrenalectomy (ADX), or both (ORX + ADX) on progesterone levels in multiple male mouse tissues. As expected, intratissue androgen levels were mainly testicular derived. Interestingly, progesterone levels remained high after ORX and ORX + ADX with the highest levels in white adipose tissue and in the gastrointestinal tract. High progesterone levels were observed in mouse chow and exceptionally high progesterone levels were observed in food items such as dairy, eggs, and beef, all derived from female animals of reproductive age. To determine if orally ingested progesterone contributes to tissue levels of progesterone in males, we treated ORX + ADX and sham mice with isotope-labeled progesterone or vehicle by oral gavage. We observed a significant uptake of labeled progesterone in white adipose tissue and prostate, suggesting that dietary progesterone may contribute to tissue levels of progesterone. In conclusion, although adrenal-derived progesterone contributes to intratissue progesterone levels in males, nonadrenal progesterone sources also contribute. We propose that dietary progesterone is absorbed and contributes to intratissue progesterone levels in male mice. We speculate that food with high progesterone content could be a significant source of progesterone in males, possibly with consequences for men undergoing androgen deprivation therapy for prostate cancer.

Key Words: progesterone, androgens, adrenalectomy, gonadectomy, gas chromatography-tandem mass spectrometry, diet

Introduction

Progesterone levels in men have been identified as a potential predictor for prostate cancer treatment effect (1, 2). Serum progesterone levels increase in prostate cancer patients receiving the cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1)-inhibitor abiraterone, which blocks local androgen production from adrenal precursors, and patients with higher progesterone serum levels after 3 months of abiraterone treatment had worse treatment outcomes. The oncogenic effect of progesterone in prostate cancer cell lines was partly androgen receptor dependent (2). Progesterone can locally, in tissues, be metabolized into the androgens testosterone and dihydrotestosterone (DHT) (3, 4) (Fig. 1). Accumulating evidence has shown that intratumoral production of DHT drives castration-resistant prostate cancer (5). Treatment with abiraterone hinders disease progression for some time, but resistance eventually occurs (6). In abiraterone-resistant prostate cancer cell lines, progesterone contributes to the activation of the androgen receptor (7).

The physiological roles of progesterone in males are still not fully known, but serum levels in men equal those in postmenopausal women (8, 9). Progesterone is the most abundant sex steroid in orchiectomized (ORX) male mice, and we have reported slightly higher progesterone levels in ORX mice compared to intact mice (10). Progesterone is generally described as being produced in both adrenals and gonads of men (8, 11). Yet, the relative importance of the gonads and adrenals for progesterone levels in males has, to the best of our knowledge, not previously been thoroughly investigated.

We have previously quantified high local levels of androgens, especially DHT, in the intestinal contents of mice of both sexes as well as in healthy men (12). In both mice and

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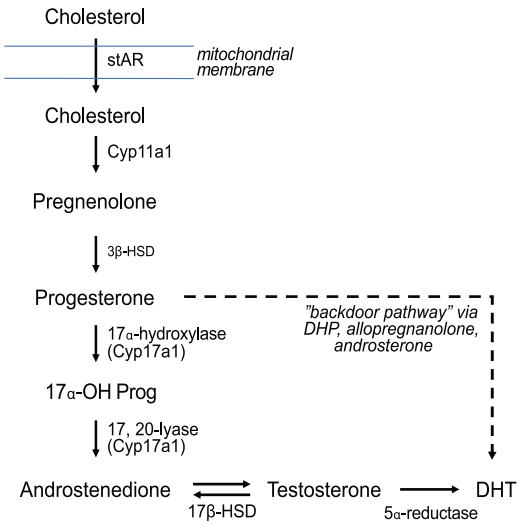


Figure 1. Schematic and simplified presentation of progesterone and androgens and their metabolic relationships.

Abbreviations: Cyp, cytochrome P450; DHP, dihydroprogesterone; DHT, dihydrotestosterone; HSD, hydroxysteroid dehydrogenase; OH prog, hydroxyprogesterone; StAR, steroidogenic acute regulatory protein.

men, testicles are the main source of testosterone, the main circulating androgen that can locally be transformed into the more potent androgen DHT (Fig. 1). The human adrenal gland also produces androgen precursors such as dehydroepiandrosterone (13-15) and androstenedione, which can be converted to active sex steroids in peripheral tissues (16). In mice, adrenals produce other androgen precursors and contribute to intratumoral levels of DHT promoting the growth of prostate cancer xenografts in the absence of gonadal androgen production (5, 17). It has also been shown that castration-resistant prostate cancer utilizes alternative pathways for intratumoral synthesis of DHT via pathways that bypass testosterone entirely (18).

The primary aim of the present study was to determine the origins of progesterone in male mice; the secondary aim was to determine the origins of intestinal and local androgens. To this end, we have subjected male mice to either adrenalectomy (ADX), ORX, or both (ORX + ADX) and measured progesterone, testosterone, and DHT in serum, multiple peripheral tissues, and different parts of the gastrointestinal tract, comparing it to controls. As we found surprisingly high remaining levels of progesterone in tissues and intestinal contents after ORX + ADX, we evaluated hormone levels in mouse chow. Then, to functionally evaluate if the high progesterone levels found in the diet could contribute to the levels found in WAT of ORX + ADX mice, we administered stable isotope-labeled progesterone or vehicle by oral gavage and analyzed labeled progesterone uptake in tissues.

Methods

Animals and Surgeries

Male C57BL/6NRj mice (11 weeks old) were purchased from Janvier Labs (Le Genest St Isle, France) and allowed to acclimatize for at least 5 days before surgery. Mice were kept in individually ventilated cages in a standard animal facility with a 12-hour light/12-hour dark cycle with free access to chow pellets (RM3(E), Special Diets Services, Essex, UK) and tap water. After ADX the mice received 0.9% saline instead of water to maintain sodium balance.

Mice were randomly divided into 4 groups: intact (control, n = 10), ADX (n = 15), ORX (n = 10), and ORX + ADX (n = 12). The ORX + ADX group was adrenalectomized 3 weeks before sacrifice, and the remaining surgeries (all orchiectomies and adrenalectomies of the ADX group) were performed 2 weeks before sacrifice and tissue collection. Mice were individually housed starting 2 weeks before sacrifice in order to avoid fighting in the cages. In the control group, 3 individuals were accidentally cohoused, and they were excluded from analysis.

When isotope-labeled progesterone was used, mice were subjected to ORX and ADX or sham surgery 2 weeks before sacrifice. After 2 days of post-surgical recovery, they received daily by oral gavage labeled progesterone (Progesterone-2,3,4,20,21-$^{13}C_5$, Sigma-Aldrich, St. Louis MO, USA) 3000 ng/day (n = 5 for ORX + ADX, n = 7 for sham operated) or vehicle only (Phosal PG 50, Lipoid, Ludwigshafen, Germany; n = 6 for ORX + ADX, n = 7 for sham operated) until the end of the study. The mice in this study were cohoused within the groups for animal welfare reasons.

Surgeries were performed under isoflourane anesthesia. The mice received preoperative analgesia buprenorphine 0.05 to 0.1 mg/kg (Temgesic, Individor Europe, Dublin, Ireland) and either carprofen (Rimadyl, Zoetis, Helsinki, Finland) or meloxicam (Metacam, Boehringer Ingelheim, Copenhagen, Denmark) subcutaneously. Carprofen or meloxicam was also given for up to 3 days postoperatively. Mice in the main experiment were fasted for 3 hours prior to sacrifice to enable sampling of bile. Bile was withdrawn using a 1 mL insulin syringe before the dissection of the liver. At sacrifice, the absence of adrenal tissue in ADX mice was confirmed, and animals with suspected remnants of adrenal tissue were excluded from the analyses (1 from the ORX + ADX group, 4 from the ADX group, none from the oral progesterone experiment). Tissues, bile content, and content from different parts of the gastrointestinal tract (stomach, small intestine, and cecum) were snap frozen in liquid nitrogen and stored at −80°C until analysis. Blood was collected from the axillary vein and allowed to coagulate at room temperature for at least 30 minutes and then centrifuged to separate the serum, which was then frozen. As the gall bladder was not filled in all animals and due to sampling errors, a sufficient amount of bile for analysis (>10 μL) was only available from 25/39 animals. The experiments were conducted in accordance with all relevant legislation and approved by the Finnish National Animal Experiment Board (ethical permission no. 5239/04.10.07/2017) or the Ethical Committee for Animal Research in Gothenburg, Västra Götaland (ethical permission no. 5.8.18-11628/2019).

Tissues and serum from germ-free and conventionally raised 8-week-old C57BL/6NTac male mice (n = 10 per group) were purchased from Taconic (Germantown, NY, USA). These mice had free access to autoclaved NIH-31 M diet.

Sex Steroid Analysis

Sample Preparation

Frozen samples were thawed on ice. Samples from tissues, intestinal contents, and food were weighed and placed in 2 mL

Table 1. Sex steroid levels in different mouse diets

Vendor	Diet	Fat[a]	Animal fat[a]	Fat sources[a]	Progesterone	Testosterone	DHT
		%	%		pg/g	pg/g	pg/g
Research Diets	D12492i	34.8	31.6	Lard, soybean oil	17 702 (183)	894 (23)	42 (5)
Research Diets	D12492	34.8	31.6	Lard, soybean oil	13 440 (304)	111 (14)	ND
Research Diets	D12451	23.6	20.6	Lard, soybean oil	9 072 (36)	115 (22)	ND
Lantmännen	R34 Vitamin E aspirin	4	0	Soybean oil	8 540 (1 402)	520 (7)	205 (15)
Plexxicon	PLX + drug	Unknown	Unknown		7 709 (303)	652 (9)	ND
Lantmännen	R34 + betacarotene	4	0	Soybean oil	7 654 (597)	686 (25)	830 (136)
Research Diets	D12450Bi	4.3	1.9	Soybean oil, lard	7 262 (83)	721 (29)	37 (6)
Lantmännen	R34 aspirin	4	0	Soybean oil	6 354 (618)	744 (37)	296 (15)
Envigo	2914C Teklad Global	4	0	Soybean oil	6 334 (268)	793 (54)	257 (20)
SAFE	U8959 version 0001	5	~ 2	Lard, colza oil, corn oil	6 135 (34)	127 (24)	ND
Envigo	2916 Teklad Global batch A	4	0	Soybean oil	5 791 (954)	526 (26)	343 (37)
Special Diet Services	RM3(E) batch B	4.3	0	Soybean oil	5 374 (177)	849 (39)	308 (71)
Envigo	2916 Teklad Global batch B	4	0	Soybean oil	5 122 (419)	636 (40)	89 (18)
Lantmännen	R34 + vitamin E	4	0	Soybean oil	4 642 (467)	712 (59)	405 (38)
SAFE	U8978 version 0022	7	0	Soybean oil	4 520 (27)	ND	ND
Plexxicon	PLX control	Unknown	Unknown		4 067 (70)	105 (13)	ND
Research Diets	D12331	35.9	0	Coconut oil, soybean oil	3 916 (209)	87 (11)	ND
SAFE	150	No added fat	0		3 708 (964)	310 (34)	ND
SAFE	A04	No added fat	0		3 401 (627)	454 (19)	ND
Special Diet Services	RM3(E) batch A	4.3	0	Soybean oil	2 878 (54)	284 (17)	547 (99)
Research Diets	D12328	4.8	0	Soybean oil, coconut oil	2 707 (85)	54 (12)	ND
Lantmännen	R34	4	0	Soybean oil	2 319 (144)	304 (10)	214 (23)
Concentrations presented as mean (SEM) from at least triplicate samples					**Mean**	**442 (63)**	**166 (47)**

Abbreviations: DHT, dihydrotestosterone; ND, not detectable.
[a]Information on fat content is taken from vendors or calculated/approximated from available documentation.

screw-top Eppendorf tubes with 450 µL PBS and homogenized by shaking with a 5 mm steel bead in a Tissuelyzer II (Qiagen, Hilden, Germany) for 5 minutes. Serum and bile samples were measured volumetrically by pipetting.

Mouse diets for steroid measurements were donated from colleagues and vendors. The diets included plant-based phytoestrogen-free diets, high-fat diets, purified diets, open-source diets, and custom diets with nonhormonal additives (Table 1). Samples of human foods were purchased at a local supermarket in Gothenburg, Sweden (Table 2).

Deglucuronidation

We have previously developed and validated a gas chromatography tandem mass spectroscopy (GC-MS/MS) assay that measures androgens and progesterone with high specificity (10, 12, 19). To assess glucuronidated forms of androgens in bile and small intestine, we compared free levels of each hormone with and without enzymatic deglucuronidation as previously described (12). Briefly, samples were divided into 2 aliquots before homogenization, and 1 of the aliquots from each sample was deglucuronidated by adding 50 µL ß-glucuronidase (from E.coli K 12 in 50% glycerol solution; Roche, Basel, Switzerland), followed by brief vortexing and incubating at 37°C with agitation for 60 minutes. Samples were then frozen at −80°C until steroid extraction and analysis. The difference between total and free levels was calculated at each site and labeled glucuronidated (gluc).

Sex Steroid Extraction and Analysis

Steroids were extracted, derivatized, and measured as described previously (10, 12, 19). Briefly, after addition of isotope-labeled internal standards, steroids were extracted by liquid-liquid extraction with 1-chlorobutane, followed by solid phase extraction using Silica SPE columns (Hypersep Si 500 mg; Thermo Fisher Scientific, Waltham, MA, USA) that were washed with ethylacetate-pentane-heptane [10:45:45

Table 2. Sex steroid levels in different human food items

Product	Progesterone	Testosterone	DHT
Butter	192 453 (21 812)		
Egg yolk	150 401 (60 926)	416 (74)	39 (3)
Cream	123 162 (1 282)		
Egg white	21 512 (600)		
Ground beef	13 876 (886)		
Cow's milk	6 362 (27)		
Sunflower seeds	421 (155)		
Castor oil	568 (32)		
Pork fat	256 (16)		
Chickpeas	201 (16)		
Pasta	172 (2)		
Potatoes			
Extra virgin olive oil			
Chicken thigh fillet			
Salmon fillet			
Walnuts			
Cod fillet			

Abbreviations: DHT, dihydrotestosterone.
Concentrations are measured in at least triplicate and presented as mean (SEM) pg/g. Empty cells indicate not detectable levels.

Table 3. Sensitivity of the gas chromatography-tandem mass spectroscopy assay in different matrices

LLOQ (pg/g)	Progesterone	Testosterone	DHT
Muscle (lean tissue)	75	20	8.0
Liver	75	40	4.0
Adipose tissue	75	40	4.0
Intestinal contents		40	20
Serum (pg/mL)	74	8	2.5

Abbreviations: DHT, dihydrotestosterone; LLOQ, lower limit of quantification.
Summarized from previous publications (10, 12, 19).

(vol:vol)]. Next, the analytes were eluted to isooctane and the organic solvent evaporated. Finally, derivatization was performed in 2 steps: oximation with pentafluorobenzylhydroxylamine hydrochloride followed by esterification with pentafluorobenzoyl chloride. Progesterone, DHT, and testosteronewere separated by gas chromatography and detected simultaneously with negative chemical ionization by an Agilent 7000 triple quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) operating in multiple reaction monitoring mode with ammonia as reagent gas; mass transitions are reported in Supplementary Table 1 (20). Peaks were integrated using the MassHunter quantitative analysis workstation software from Agilent. Measured concentrations were corrected for amount of input material (wet mass of intestinal contents and tissues or volume of serum and bile). The method has been validated in serum, different types of tissues (lean, adipose tissue, and liver, which is a complex and metabolite-rich matrix), and intestinal contents (10, 12, 19, 21). A description of the validation experiments can be found in the supplementary materials (20). The lower limits of

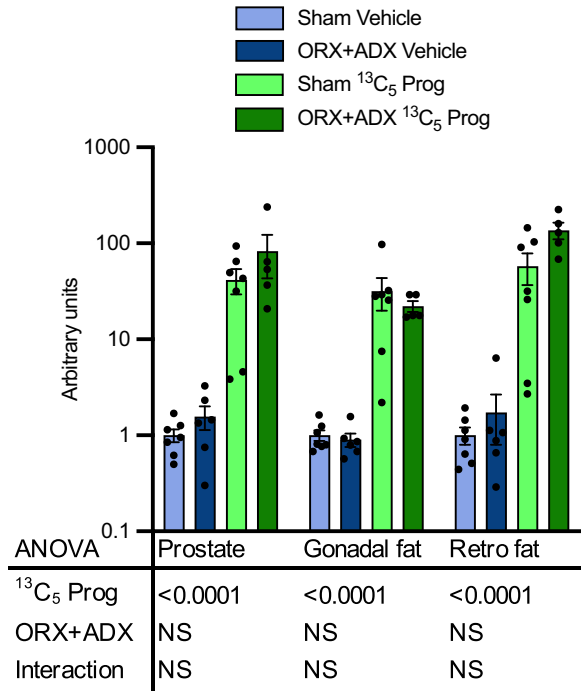
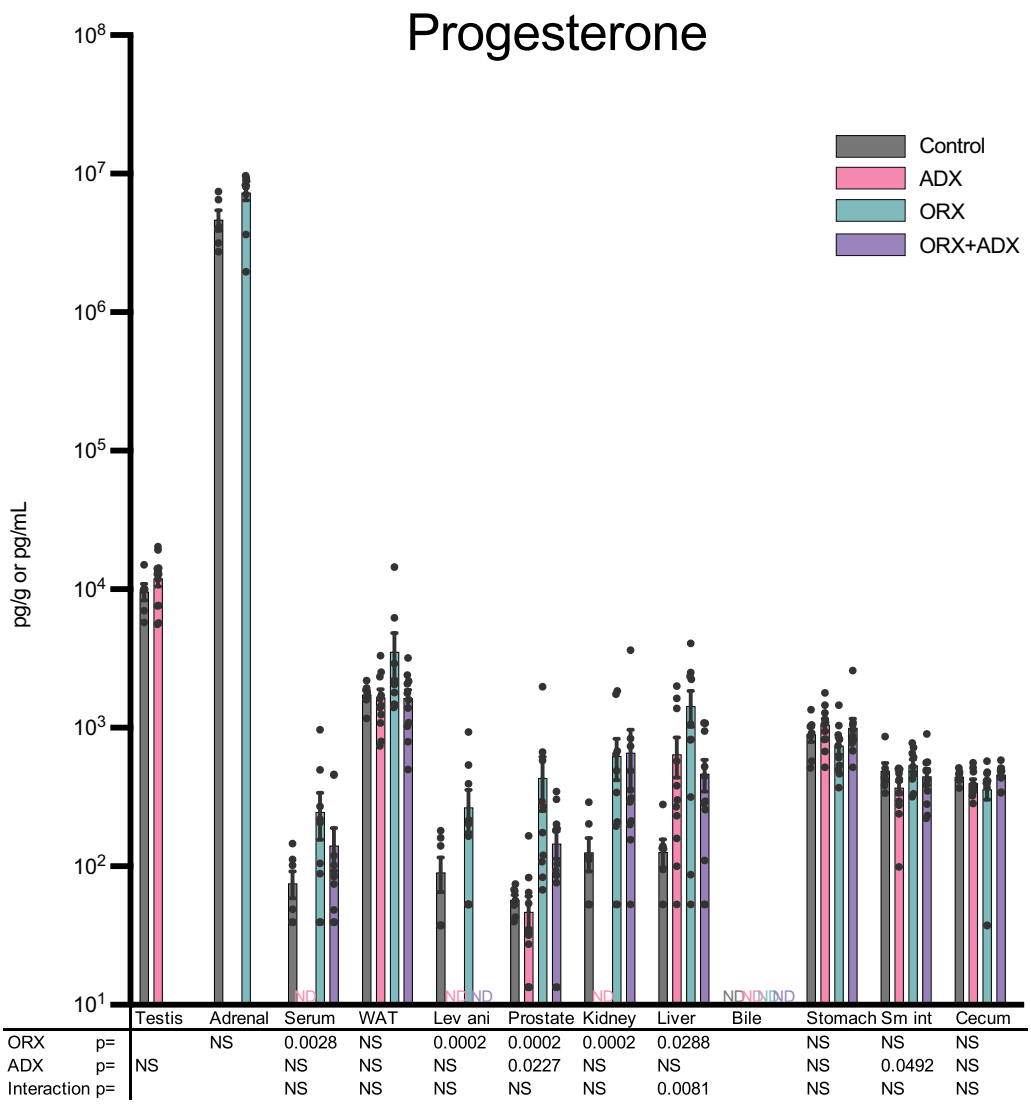


Figure 2. Uptake of orally administered labeled progesterone in tissues. Orchiectomized and adrenalectomized (ORX + ADX) or sham operated male C57Bl6/NRj mice were treated by oral gavage with either stable isotope-labeled progesterone ($^{13}C_5$-Prog; n = 5 for ORX + ADX, n = 7 for sham) or vehicle (n = 6 for ORX + ADX; n = 7 for sham) for 11 days. The area of the response from the gas chromatography/tandem mass spectroscopy was divided with the mass of input material, followed by normalization of all values to the mean for the vehicle group for that tissue. Bars represent the mean with error bars representing the standard error of the mean. Scatters represent individual values. Two-way ANOVA (treatment, surgery, and their interactions) for each tissue after log-transformation of the values.

Abbreviations: NS, not significant; Retro, retroperitoneal.

quantification (LLOQs) for the reported analytes were published previously and are summarized in Table 3. For the prostate, the sensitivity of the assay for progesterone was improved compared with our previous publication to an LLOQ of 19 pg/g. For the analysis of $^{13}C_5$-progesterone, the mass units for multiple reaction monitoring were adjusted for the higher mass provided by the additional ^{13}C atoms. In these measurements, no internal standard was used as the $^{13}C_3$-progesterone normally used as internal standard was found to interfere with measurement of $^{13}C_5$-progesterone. In vehicle-treated mice, a baseline reading of $^{13}C_5$-progesterone was seen, reflecting the natural presence of carbon 13. To evaluate the presence of labeled progesterone, we performed 2 independent calculations: first, the uncorrected GC-MS/MS-area for $^{13}C_5$-progesterone per input mass of tissue was determined. The area per input mass was normalized so that the mean value of the control group was 1 and the results were reported as arbitrary units (Fig. 2). In a second independent calculation, to estimate the concentration of labeled progesterone in relation to the levels of endogenous progesterone, the area for the labeled progesterone was divided by the area for the unlabeled progesterone in the same analysis as a replacement for an internal standard (Supplementary Fig. S1) (20).

In mouse tissues and serum, values below the LLOQ were set to LLOQ/$\sqrt{2}$ in order not to overestimate undetectable



		Testis	Adrenal	Serum	WAT	Lev ani	Prostate	Kidney	Liver	Bile	Stomach	Sm int	Cecum
ORX	p=		NS	0.0028	NS	0.0002	0.0002	0.0002	0.0288		NS	NS	NS
ADX	p=	NS		NS	NS	NS	0.0227	NS	NS		NS	0.0492	NS
Interaction	p=			NS	NS	NS	NS	NS	0.0081		NS	NS	NS

Figure 3. Progesterone levels in tissues and gastrointestinal contents of male mice subjected to ADX and/or ORX. Male C57Bl6/NRj mice were subjected to ADX (n = 11), ORX (n = 10), or both (ADX + ORX, n = 11), and local progesterone levels were measured using gas chromatography/tandem mass spectroscopy 2 weeks after last surgery, compared to control mice (n = 7). All values represent free, unconjugated progesterone. Two-way ANOVA (ADX, ORX, and their interactions) for each tissue after log-transformation of the values. Bars represent the mean with error bars representing the standard error of the mean. Scatters represent individual values.

Abbreviations: ADX, adrenalectomy; Lev ani, levator ani muscle; ND, not detectable; NS, not significant; ORX, orchiectomy; sm int, small intestine; WAT, white adipose tissue.

hormone levels (22). To calculate the levels of glucuronidated steroids in small intestine and bile, the free level (concentration without glucuronidation) was subtracted from the total level (concentration after deglucuronidation) as described previously (12). One gram of tissue or intestinal contents was considered equivalent to 1 milliliter of serum or bile. In mouse chow and food, measurements below the lowest point of the standard curve or below the LLOQ in tissues were denoted as not detected.

Gene Expression Analysis

RNA was isolated from gonadal fat using TriZol reagent (Thermo Fisher Scientific) followed by RNeasy Mini Kit (Qiagen). The RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR analyses were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) and Taqman probes (Thermo Fisher Scientific), labeled with the reporter fluorescent dye FAM (*Star*: Mm00441558_m1, *Cyp11a1*: Mm00490735_m1, *Hsd3b1*: Mm00476184_g1, *Hsd3b2*: Mm00462683_g1, *Hsd3b3*: Mm001729523_m1, *Hsd3b6*: Mm00834440_m1). As an internal standard, predesigned primers and probe labeled with the reporter fluorescent dye VIC, specific for 18S ribosomal RNA (4310893E, Applied Biosystems), were included in the reactions. The gene expression was calculated using the ΔΔCT method with 18S as reference gene.

Statistics

Graph Pad Prism 9 (Graph Pad Software, San Diego, CA, USA) and Microsoft Excel 16 (Microsoft, Redmond, WA, USA) were used for statistics. Sex steroid levels were log

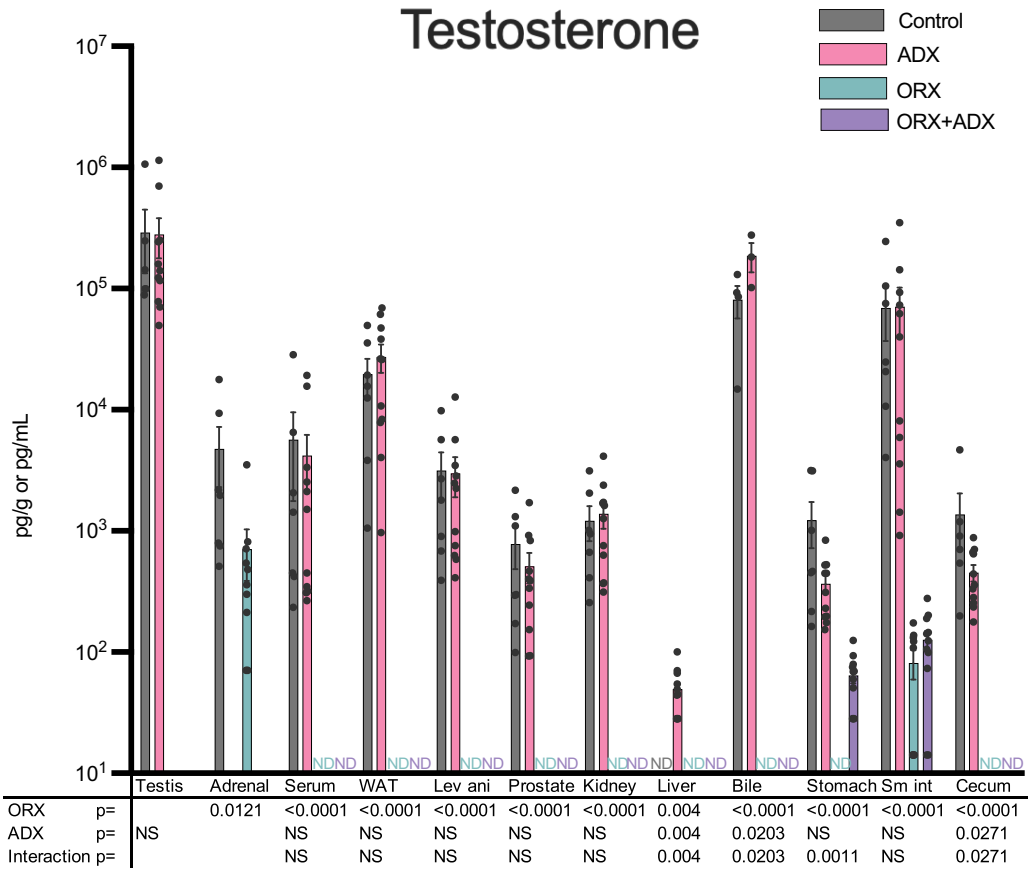


Figure 4. Testosterone levels in tissues and gastrointestinal contents of male mice subjected to ADX and/or ORX. Male C57Bl6/NRj mice were subjected to ADX (n = 11), ORX (n = 10), or both (ADX + ORX, n = 11), and local testosterone levels were measured using gas chromatography/tandem mass spectroscopy 2 weeks after last surgery, compared to control mice (n = 7). The values in bile and small intestine represent glucuronidated testosterone (expressed as concentration testosterone); all other values represent free testosterone. Two-way ANOVA (ADX, OR,X and their interactions) for each tissue after log-transformation of the values. Bars represent the mean with error bars representing the standard error of the mean. Scatters represent individual values.

Abbreviations: ADX, adrenalectomy; Lev ani, levator ani muscle; ND, not detectable; NS, not significant; ORX, orchiectomy; sm int, small intestine; WAT, white adipose tissue.

transformed. The effect of ORX and ADX and their interaction factor was evaluated using two-way ANOVA. Comparison of 2 groups was done by Student's t-test. Comparison of the effect of treatment and surgeries in the labeled progesterone study was done using two-way ANOVA. The correlation analysis in the diet was performed using Pearson correlation.

Results

To determine the origins of progesterone and androgens in male mice, we first analyzed sex hormone levels in serum, liver, prostate, seminal vesicles, white adipose tissue (WAT; gonadal fat), kidney, testicles, adrenals, and the levator ani muscle (m. levator ani) as well as contents of the gastrointestinal tract (stomach, small intestine, cecum, and bile) with and without ORX and/or ADX.

Progesterone was detected in most tissues with substantial levels remaining after ORX and ADX (Fig. 2). In WAT, progesterone levels were higher than serum levels and progesterone levels in WAT after ORX + ADX were similar to those in intact mice (1630 pg/g ± 239 vs 1871 pg/g ± 124; mean ± SEM) (Fig. 3). In the adrenals, progesterone concentrations were substantially higher than in all other investigated tissues both with and without ORX, supporting the notion that progesterone may be secreted from the adrenals. Adrenalectomy did not significantly affect progesterone levels at most sites. In the gastrointestinal tract, progesterone levels started at around 800 pg/g in the stomach and decreased slightly to around 500 pg/g in small intestine and cecum with no effect from ORX or ADX (Fig. 3). Progesterone increased after ORX in the prostate and in the sex steroid-metabolizing/excreting organs, liver and kidney. No progesterone was detected in the bile (Fig. 3). As progesterone levels remained essentially unchanged in several parts of the body after ORX + ADX, another source of progesterone other than the gonads or adrenals must exist.

Testosterone and DHT concentrations were undetectable or significantly reduced in most sites after ORX with or without ADX (Figs. 4 and 5). After ORX, testosterone was reduced in the peripheral tissues WAT, prostate, seminal vesicles, m. levator ani, and kidney to near or below the LLOQ (Fig. 4). The potent androgen DHT decreased after ORX to 1/50 to 1/200 in prostate, WAT, cecum, and m. levator ani, with levels after ORX close to or below the LLOQ in serum, liver, kidney, and bile (Fig. 5). All of the testosterone and DHT detected in the bile was glucuronidated, and the majority of the testosterone and DHT detected in

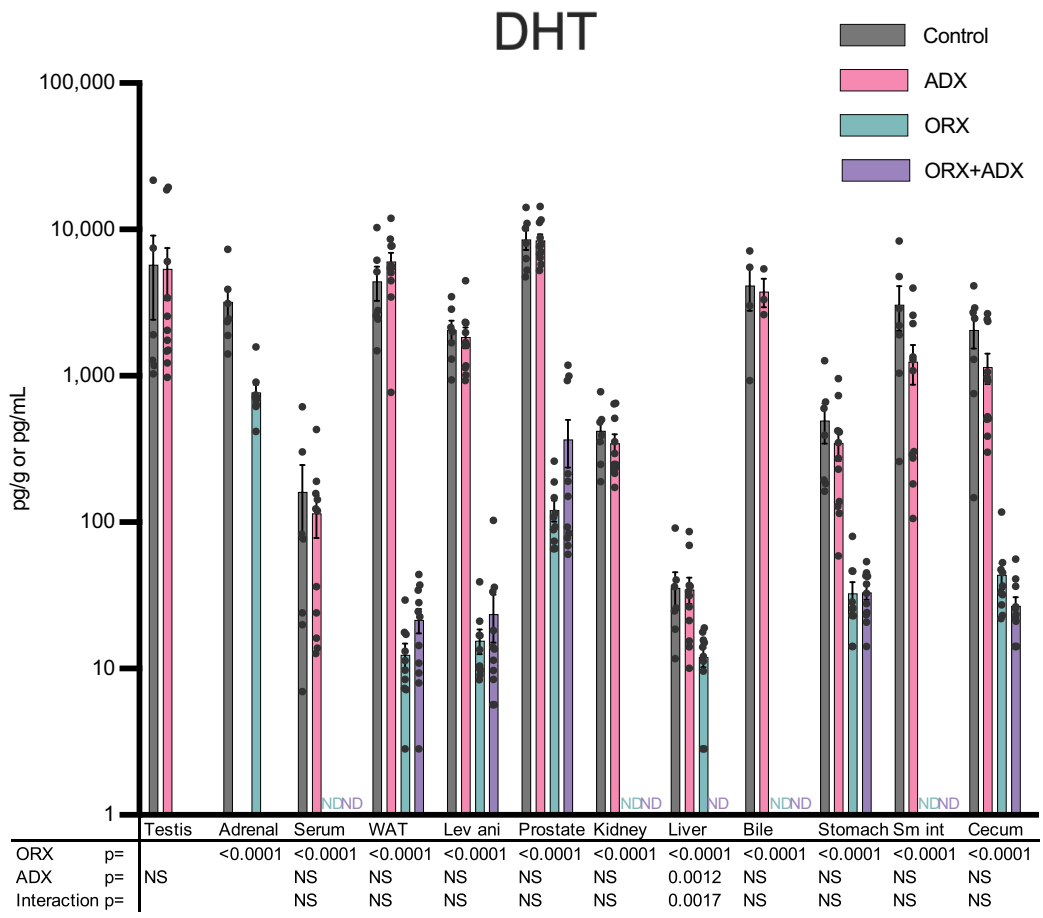


Figure 5. DHT levels in tissues and gastrointestinal contents of male mice subjected to adrenalectomy and/or orchiectomy. Male C57Bl6/NRj mice were subjected to adrenalectomy (ADX, n = 11) orchiectomy (ORX, n = 10) or both (ADX + ORX, n = 11) and local DHT levels were measured using gas chromatography/tandem mass spectroscopy two weeks after last surgery, compared to control mice (n = 7). The values in bile and small intestine represent glucuronidated DHT (expressed as concentration DHT), all other values represent free DHT. Two-way ANOVA (ADX, ORX and their interactions) for each tissue after log-transformation of the values. Bars represent the mean with error bars representing the standard error of the mean. Scatters represent individual values.

Abbreviations: ADX, adrenalectomy; DHT, dihydrotestosterone; Lev ani, levator ani muscle; ND, not detectable; NS, not significant; ORX, orchiectomy; sm int, small intestine; WAT, white adipose tissue.

the small intestine was glucuronidated (Supplementary Table 2) (20). In intact males, the concentration of free DHT in cecum was similar to the concentration of glucuronidated DHT detected in the bile and small intestine, indicating that the majority of DHT found in the intestine originated from the circulation and was excreted via the bile into the intestine. Interestingly, low but measurable concentrations of DHT were present in the prostate, m. levator ani, and WAT in mice lacking both testes and adrenals (Fig. 5). Thus, we did not detect any significant adrenal contribution to the levels of testosterone or DHT in serum or most tissues.

As we found that progesterone levels were not majorly affected by ORX and/or ADX and progesterone levels were high in the gastrointestinal tract content, we hypothesized that the diet might be a source of intraintestinal progesterone. Analyses of the sex steroid content of the mouse chow used in the present study revealed high levels of progesterone (2878 ± 54 pg/g; 50x the serum levels in male mice) and rather low levels of androgens (Fig. 6; Table 1). To understand the generalizability of this finding, we also tested multiple other brands of mouse diets. All the sampled diets contained progesterone with concentrations between 2319 and 17702 pg per gram diet (mean 6302 pg/g). The level of progesterone correlated

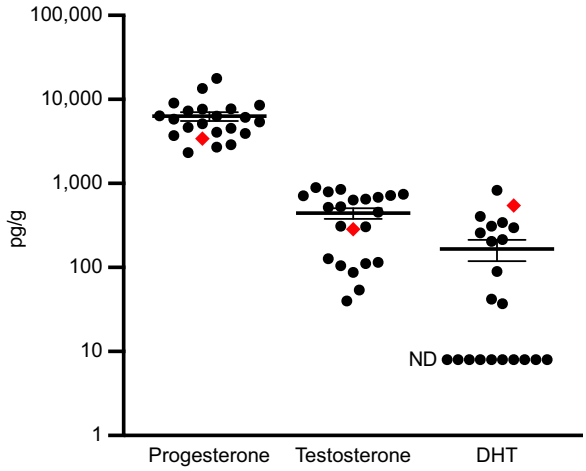


Figure 6. Sex steroid contents in 22 different mouse chow samples measured using gas chromatography/tandem mass spectroscopy. Each sample was measured in at least triplicate, and the mean is given. The red diamond represents the measurement from the diet used in the current study.

Abbreviations: DHT, dihydrotestosterone; Prog, progesterone; T, testosterone.

Table 4. Expression of hormone-producing enzymes in gonadal fat

	Relative expression				ANOVA		
	Control	ADX	ORX	ADX + ORX	Surgery	Treatment	Interaction
stAR	1.94 (0.18)	1.36 (0.18)	2.14 (0.22)	1.91 (0.28)	NS	NS	NS
Cyp11a1	2.10 (0.38)	2.15 (0.48)	2.41 (1.07)	1.32 (0.20)	NS	NS	NS
HSD3b1	ND	ND	ND	ND			
HSD3b2	ND	ND	ND	ND			
HSD3b3	ND	ND	ND	ND			
HSD3b5	50.5 (39.7)	85.6 (54.7)	1.13 (0.74)	1.41 (0.41)	NS	NS	NS
HSD3b6	ND	ND	ND	ND			

Abbreviations: ADX, adrenalectomized; Cyp, cytochrome P450; HSD, hydroxysteroid dehydrogenase; ND, not detected; NS, not significant; ORX, orchiectomized; stAR, steroidogenic acute regulatory protein.
Arbitrary units, delta deltaCT method corrected for the expression of 18S presented as mean (SEM). Significances for each gene according to two-way ANOVA.

with the amount of animal-derived fat (lard) in the diets (Pearson correlation coefficient r = 0.79; $P < .001$), but significant amounts were also found in diets declared to contain only vegetable fats (Table 1). All diets contained added vitamins and minerals. The observation of high progesterone levels in different mouse diets was confirmed by an independent liquid chromatography mass spectrophotometric analytical method (9), yielding similar results as the GC-MS/MS method used in the present study (data not shown). In a selection of human food items, we found exceptionally high levels (>100-fold higher than male mouse tissue levels) of progesterone in dairy products, beef, and eggs, all derived from female animals at reproductive age (Table 2).

To mechanistically evaluate if the dietary progesterone could be a source of progesterone in male mice, we administered stable isotope-labeled progesterone ($^{13}C_5$-progesterone) or vehicle by gavage to ORX + ADX and sham operated mice for 11 days. In WAT (both gonadal and retroperitoneal fat depots) and prostate, oral treatment with $^{13}C_5$-progesterone substantially increased the presence of $^{13}C_5$-progesterone in the tissues both when comparing weight-adjusted raw areas (Fig. 2) and when analyzing the areas in relation to the area for native progesterone (Supplementary Fig. 1) (20). The concentration of labeled progesterone in prostate and both types of WAT was around 10% of the concentration of native progesterone in treated ORX + ADX mice and around 1% in treated sham mice, suggesting that the contribution of orally ingested progesterone to intratissue levels of progesterone may be more pronounced after ADX (Supplementary Fig. 1) (20). As our data demonstrated that orally ingested progesterone contributes to tissue levels of progesterone in WAT, we evaluated if the gut microbiota may regulate progesterone uptake in male mice by comparing germ-free mice with conventionally raised mice. Serum levels of progesterone were not different (490 ± 256 pg/g vs 424 ± 176 pg/g; mean ± SEM), but in WAT, levels were higher (+ 142%; 11 155 ± 1 193 pg/g vs 4 607 ± 968 pg/g; $P = .0003$) in germ-free mice compared to conventionally raised mice.

Finally, to evaluate if local progesterone production in adipose tissue could be another source of progesterone in WAT of male mice, we analyzed gene expression of steroidogenic enzymes upstream of progesterone (*StAR*, *Cyp11a1*, *Hsd3b1-3,5-6*) (Fig. 1). *StAR* and *Cyp11a1* were expressed in WAT but were not regulated by ADX or ORX. *Hsd3b5* had a large variability in its expression while the other *Hsd3b* isoforms were not expressed (Table 4).

Discussion

As the origin of progesterone in males is unclear and progesterone levels have been shown to predict treatment success in advanced prostate cancer patients (2), we herein evaluated the origin of progesterone and androgens in male mice by analyzing serum and tissue hormone levels after ORX, ADX, or both. We found that even though progesterone was present in very high levels in the adrenals, progesterone levels remained unchanged in several tissues after ORX + ADX. We also demonstrated that progesterone can be taken up from the diet, in which it is present in high concentrations, suggesting that dietary progesterone is a possible source of intratissue levels of progesterone in male mice.

As expected, intratissue levels of androgens were mainly testis derived, but remaining levels of DHT in prostate and several other tissues after ORX + ADX suggest that some nongonadal, nonadrenal contribution to local androgen levels do exist. In contrast to the androgens, progesterone levels in serum, tissue, and intestinal contents showed surprisingly little dependence on adrenals or gonads in male mice. The high levels of progesterone in the stomach and the high concentrations of progesterone in all analyzed variants of mouse diets suggest that dietary progesterone contributes to the progesterone levels in the intestinal contents (Fig. 7). No progesterone was detected in the bile, demonstrating that there is no bile excretion of progesterone unlike for testosterone and DHT. Thus, we hypothesized that the progesterone levels found in serum and WAT could in part originate from the substantial intake of progesterone from food. This hypothesis was functionally tested, and we could clearly detect progesterone uptake into WAT and prostate when stable isotope-labeled progesterone was given orally to male mice. In ORX + ADX mice, the proportion of labeled to unlabeled progesterone was higher than in sham mice, suggesting that the contribution of orally ingested progesterone to intratissue levels of progesterone may be more pronounced in the absence of adrenals. Germ-free male mice had higher levels of progesterone in WAT compared to conventionally raised mice, suggesting that the overall effect of the gut microbiota may be to reduce the uptake of dietary progesterone.

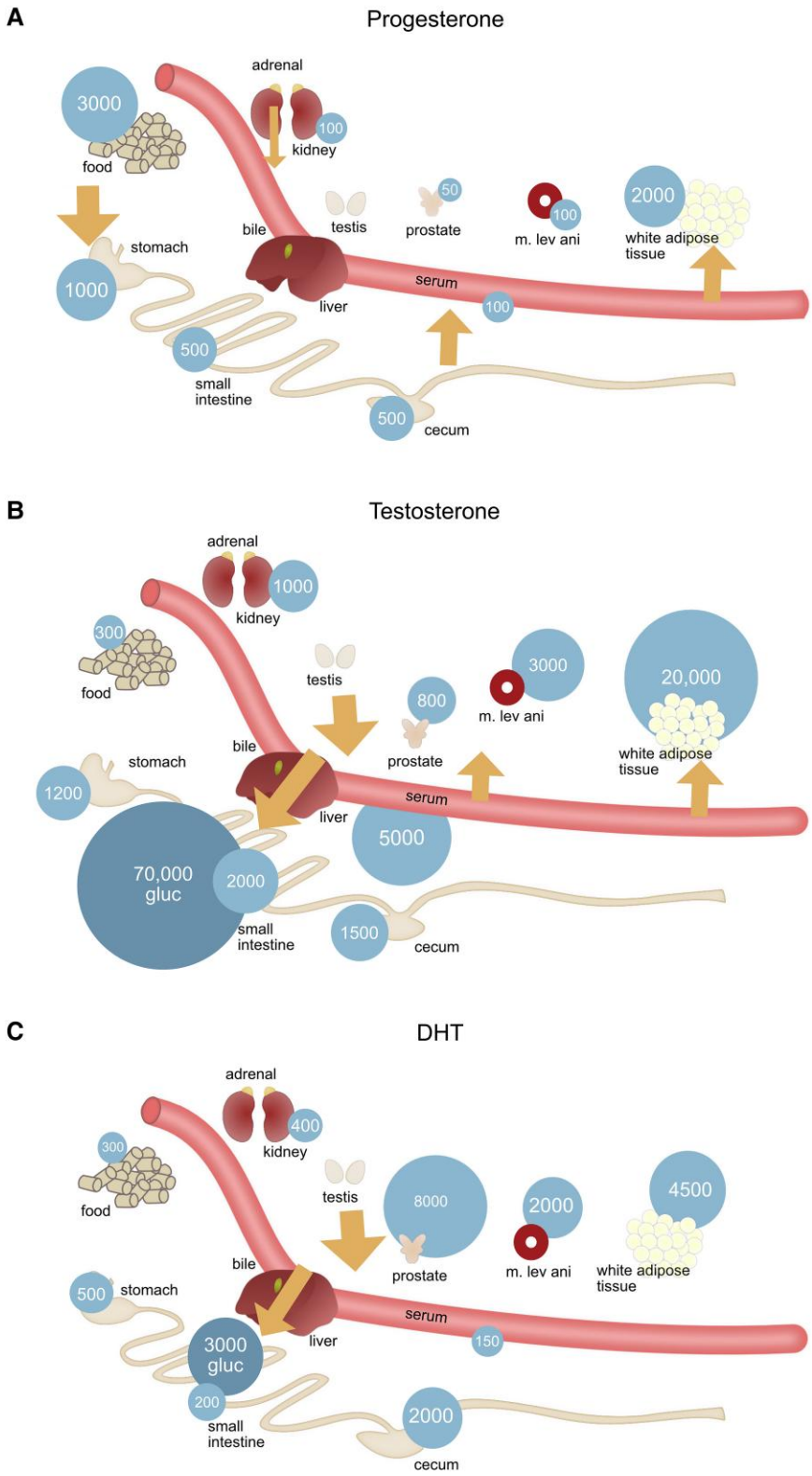


Figure 7. Visualization of sex steroid levels (light blue circles) in different body parts of intact male mice and proposed flow of the different hormones (yellow arrows) in the male mouse body. Darker blue circles represent conjugated (glucuronidated, gluc) hormones. Levels in endocrine organs (testicles and adrenals) are not shown. (A), testosterone is mainly produced in the testes and excreted into the intestine via bile; (B), DHT is locally produced for example in the prostate and dependent on testicular production of precursors; (C), progesterone originates from the adrenals and from intestinal uptake from food.

Abbreviations: DHT, dihydrotestosterone; m. lev ani, levator ani muscle.

Progesterone may contribute to growth of prostate cancer both as an androgen precursor and by nonandrogen dependent pathways (2). The androgen receptor-dependent effects of progesterone in prostate cancer cell lines have been reported to include both canonical and noncanonical androgen receptor signaling, involving activation of the transcription factor MYC (2). High MYC expression has been linked to worse prostate cancer progression and poorer treatment response in patients (23, 24). In addition, both progesterone receptor α and ß are expressed in prostate cancer tissue (25-27). High expression of progesterone receptor ß has been linked to clinical failure in prostate cancer patients (28, 29), but it is unclear if progesterone promotes prostate cancer growth via activation of progesterone receptor signaling. Thus, there are multiple possible mechanisms whereby progesterone could promote prostate cancer initiation or growth, and we speculate that these mechanisms may be affected by dietary progesterone in men.

In the present study, we observed very high progesterone levels in cow's milk and its derivatives as well as in eggs and some types of meat. This confirms previous findings (30, 31) and is in line with the high progesterone levels present in reproductive females. A possible uptake of dietary sex steroids in individuals with low endogenous sex steroids is supported by an altered urinary steroid profile after intake of cow´s milk in postmenopausal women (32) and short-term serum and urinary changes in progesterone after consumption of milk in prepubertal children (33). However, the possible uptake and bioavailability of dietary progesterone in men is unknown.

Prostate cancer and its relationship with different aspects of diet have been investigated in several studies but with equivocal results (34). In meta-analyses of prospective studies, prostate cancer risk has been linked to high intake of (certain) dairy products (35, 36), while other studies found no association of dairy intake and prostate cancer risk (37, 38). None of the studies analyzed circulating progesterone levels. In castrated mice, a low-carbohydrate diet could suppress the growth rate of prostate cancer xenografts and the intratumoral concentrations of DHT compared to a Western diet (39). The hormone levels of the 2 different diets used in that study were, however, not reported. We have, in the present study, shown that high and variable progesterone levels are present in mouse chow (and certain human food items) and that progesterone can be taken up in the intestine in mice. These findings suggest that dietary progesterone could be a factor to consider in future research on sex steroid-dependent diseases in men.

We and others have previously detected high levels of progesterone in the adrenals from different mouse strains (5, 10, 17). In some previous studies, progesterone levels in serum increased with ORX, similarly as seen in the present study, and decreased modestly with ADX (5, 17). Local progesterone levels in the prostate, liver, and kidney increased after ORX in the present study. We propose that the modest increase in progesterone levels observed after ORX (by us and others) may depend on the lower metabolism of progesterone as an androgen precursor when the testicles are removed. Another hypothesis is that ORX increases the adrenal weight and upregulates adrenal steroidogenic enzymes, thereby increasing adrenal progesterone production (5, 10). However, this does not seem to be the case in this study as the increase was observed even in ORX + ADX mice.

Gene expression analyses showed that there was no significant upregulation of progesterone-producing enzymes in WAT after ORX or ADX, indicating that increased local production in adipose tissue is not a major contributor to the remaining progesterone levels in ORX + ADX male mice.

In the present study, the systemic androgens testosterone and DHT originated mostly from the testicles. The presence of more glucuronidated testosterone and DHT in the bile and small intestine of gonadal intact mice than in ORX mice indicates that these hormones are secreted from the systemic circulation into the intestine, confirming the results of our previous study (12). Interestingly, low but measurable concentrations of DHT were present in the prostate, m. levator ani, and WAT in ORX + ADX mice in the present study. It is possible that the remaining high local progesterone levels in ORX + ADX mice might act as a precursor for the remaining DHT levels in these tissues. Indeed, expression of steroidogenic enzymes downstream of progesterone has been previously shown in both prostate (40, 41) and adipose tissue (42).

The present study has several strengths. We have used a highly sensitive and specific validated GC-MS/MS-method to determine sex steroid levels in multiple different matrices. This is the first study to analyze progesterone and androgens in different parts of the intestinal tract in ORX and ADX mice, as well as in mouse chow using the same GC-MS/MS methodology. We used stable isotope-labeled progesterone to mechanistically determine the possibility of uptake of dietary progesterone into tissues. Weaknesses of the current study include that it was not possible to directly quantify the labeled progesterone uptake, only relatively, due to interference between $^{13}C_3$-progesterone normally used as internal standard and the dosed $^{13}C_5$-progesterone. The measured hormone levels of highly vascularized tissues with low hormone levels may have been influenced by the hormone levels in the blood contained in the tissues at sacrifice. Further studies should evaluate the source of the progesterone found in mouse chow as well as the effect of dietary progesterone on systemic/local progesterone levels. Another area that merits further investigation is dietary progesterone's relevance as an androgen precursor or direct ligand in castration-resistant prostate cancer, especially in patients under androgen deprivation therapy.

In conclusion, although adrenal-derived progesterone is likely a major contributor to progesterone levels in males, nonadrenal progesterone sources also contribute. We propose that dietary progesterone can be absorbed and contribute to intratissue progesterone levels, of relevance especially after treatments blocking endogenous sex steroid synthesis. Also, the demonstrated presence of high levels of progesterone in many types of commonly used rodent chow could be an important factor to consider in future studies on sex steroid-dependent phenotypes. We finally propose that certain food items, derived from female animals of reproductive age, could be a substantial source of progesterone in males, possibly with consequences for men undergoing androgen deprivation therapy for prostate cancer.

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Author Contributions

Designed research: H.C., L.V., M.P., C.O.; performed research: H.C., M.H.T., A.L., K.H., J.W., K.H.N., L.G.; supervised animal experiments: M.L.; analyzed data: H.C., M.H.T., A.L., H.R., M.P., L.V., C.O.; wrote the manuscript: H.C., L.V., C.O.; revised the manuscript: M.H.T., A.L., K.H., J.W., M.L., K.H.N., L.G., M.P., H.R., L.V., C.O. All authors approved the final version of the manuscript. H.C. and C.O. take responsibility for the integrity of the data analysis.

Disclosures

The authors have nothing to disclose.

Data Availability

Some or all datasets generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

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