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A low carbohydrate, high protein diet suppresses intratumoral androgen synthesis and slows castration-resistant prostate tumor growth in mice

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ABSTRACT

Dietary factors continue to preside as dominant influences in prostate cancer prevalence and progression-free survival following primary treatment. We investigated the influence of a low carbohydrate diet, compared to a typical Western diet, on prostate cancer (PCa) tumor growth *in vivo*. LNCaP xenograft tumor growth was studied in both intact and castrated mice, representing a more advanced castration resistant PCa (CRPC). No differences in LNCaP tumor progression (total tumor volume) with diet was observed for intact mice ($P = 0.471$) however, castrated mice on the Low Carb diet saw a statistically significant reduction in tumor growth rate compared with Western diet fed mice ($P = 0.017$). No correlation with serum PSA was observed. Steroid profiles, alongside serum cholesterol and cholesteryl ester levels, were significantly altered by both diet and castration. Specifically, DHT concentration with the Low Carb diet was 58% that of the CRPC-bearing mice on the Western diet. Enzymes in the steroidogenesis pathway were directly impacted and tumors isolated from intact mice on the Low Carb diet had higher AKR1C3 protein levels and lower HSD17B2 protein levels than intact mice on the Western diet (ARK1C3: $P = 0.074$; HSD17B2: $P = 0.091$, with $\alpha = 0.1$). In contrast, CRPC tumors from mice on Low Carb diets had higher concentrations of both HSD17B2 ($P = 0.016$) and SRD5A1 ($P = 0.058$ with $\alpha = 0.1$) enzymes. There was no correlation between tumor growth in castrated mice for Low Carb diet *versus* Western diet and (a) serum insulin (b) GH serum levels (c) insulin receptor (IR) or (d) IGF-1R in tumor tissue. Intact mice fed Western diet had higher serum insulin which was associated with significantly higher blood glucose and tumor tissue IR. We conclude that both diet and castration have a significant impact on the endocrinology of mice bearing LNCaP xenograft tumors. The observed effects of diet on cholesterol and steroid regulation impact tumor tissue DHT specifically and are likely to be mechanistic drivers behind the observed tumor growth suppression.

1. Introduction

Prostate cancer is the second-most common cancer in men and the sixth-leading cause of cancer-related death world-wide [1]. These rates are typically higher for Western nations [1], and epidemiological studies have shown positive associations with rates of obesity [2–4]. Diets with a high carbohydrate content promote tumor progression in prostate cancer xenograft models [5,6] We have also demonstrated the efficacy of a low carbohydrate – high protein diet in attenuating growth of both murine squamous cell and human colorectal carcinomas in mice [7]. More recently, we reported effects of our low carbohydrate study diet tested alone and in combination with celecoxib to significantly inhibit metastases in both the 4T1 lung cancer model as well as prostate cancer metastasis in the murine TRAMP mouse model [8]. These observations suggest diet-focused prophylaxis may impede prostate carcinogenesis and also have a role in preventing progression to metastasis once cancer is established.

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Survival and proliferation of prostate cancer cells is androgen-dependent [9–10], and androgen-deprivation therapies (ADT) with agonists or antagonists of luteinizing hormone releasing hormone (LHRH) are often only temporarily effective [11,12]. This temporary remission leads to a subsequent relapse into a "castration-resistant" prostate cancer (CRPC) phenotype that remains androgen-dependent and may rely on alternative androgen sources (*e.g.*, adrenal [13]) or *de novo* intratumoral steroidogenesis [14,15]. Several lines of evidence suggest that CRPC involves reactivation of androgen-dependent pathways mediated by the androgen receptor (AR) [16,17], including a steady increase in serum levels of prostate-specific antigen (PSA), an androgen-regulated gene, after initial castration [18].

Prostate tumors express all the necessary steroidogenic enzymes for *de novo* steroidogenesis from cholesterol [19], and intratumoral cholesterol bioavailability, in the form of cholesteryl esters, increases following castration [20]. Diets with a high carbohydrate content may further increase serum cholesterol levels [21–23], and this excess may lead to increased allocation toward esterified derivatives that can be cleaved to provide free cholesterol for intratumoral steroidogenesis [20,24]. Hypercholesterolemia is associated with increased size of prostatic tumors and higher intratumoral testosterone (T) levels in gonadally intact LNCaP xenograft mice [21], increased angiogenesis [25], and a reduction in apoptosis of prostate cancer cells [26]. Conversely, hypocholesterolemia generally inhibits prostatic tumor growth [21]. Serum cholesterol levels are aligned with prostate cancer progression, and explanations for these effects include: age-related declines in the ability of prostate tissue to regulate cholesterol [27]; activation of growth factor signal transduction pathways [26]; and increased availability of substrate for local steroid synthesis [21]. Thus, diet regimes that deplete serum cholesterol may suppress CRPC tumor progression.

ADT can induce a metabolic syndrome in patients that includes both elevated fasting serum insulin and glucose levels. This effect of ADT may be enhanced by high-carbohydrate/fat diets [28], and insulin and glucose promote aggressive tumor growth in mouse models [7,29]. An *in vitro* study demonstrated that insulin treatment up-regulates several key steroidogenic enzymes and promotes androgen synthesis in LNCaP cells [30]. Furthermore, CRPC developed in LNCaP xenografted mice showed higher levels of insulin receptor (IR) and insulin receptor substrate 2 (IRS-2) [30]. Hyperglycemia further promotes growth of cancer cells, which have higher glycolytic demands than non-cancerous cells [7,31]. Taken together, these studies suggest that diets that promote metabolic syndrome have great potential to exacerbate tumor growth after ADT through a number of mechanisms.

We investigated the effects of diet on CRPC tumor growth in LNCaP xenograft mice by comparing a high-carbohydrate diet (hereafter Western diet) to a low-carbohydrate diet (hereafter Low Carb diet). Diets were made isocaloric by increasing relative protein content while maintaining approximately equivalent fat content. We predicted that CRPC in mice held on a Low Carb diet would exhibit decreased intratumoral steroidogenesis; lowered cholesterol uptake; less availability of glucose for oxidative fuel; and suppressed insulin-mediated signaling, resulting in an impaired reactivation of the AR pathway and thus a slower progression of CRPC.

2. Materials and methods

2.1. Reagents and antibodies

Western diet (TestDiet 5SGN: fat: 21.6%, protein: 23.2%, carbohydrate: 55.2%), Low Carb diet (TestDiet 5SAM: fat: 26.2%, protein: 58.2%, carbohydrate: 15.6%); antibodies: rabbit polyclonal CYP17A1 (1:1000), a gift from Dr. D.B. Hales (University of Illinois – Chicago, IL); goat polyclonal CYP11A1 (1:200), and rabbit polyclonal for StAR (1:200), fatty acid synthase (FASN, 1:2000), AR (1:100), insulin-like growth factor-1 receptor (IGF-1R, 1:25), and insulin receptor substrate-2 (IRS-2, 1:10) from Santa Cruz Technologies (Santa Cruz, CA); rabbit polyclonal for CYB5A (1:500), HSD17B2 (1:1000), and low-density lipoprotein receptor (LDL-R, 1:50), and mouse monoclonal AKR1C3 (1:2000) from Abcam (Toronto, ON); mouse monoclonal HSD3B2 (1:200), and mouse polyclonal for HSD17B3 (1:1000) and RDH5 (1:1000) from Abnova (Taipei, Taiwan); goat polyclonal SRD5A1 (1:5000) from Novus Biologicals (Littleton, CO); rabbit polyclonal IR (1:50) from EMD Milipore (Billerica, MA); and rabbit monoclonal Ki67 (1:500) from Thermo Scientific (Waltham, MA). For enzymes, all secondary antibodies used were IRDye® from LI-COR Biosciences (Lincoln, NE). Receptor immunohistochemical staining was conducted using a Ventana autostainer model Discover XT™ (Ventana Medical System, Tucson, AZ) with an enzyme labeled biotin/streptavidin system and solvent resistant DAB Map kit.

2.2. In vivo model and diet manipulation

All animal experiments were conducted in accordance with the University of British Columbia Committee on Animal Care. Athymic nude mice (Harlan Sprague Dawley, Inc.) were subcutaneously inoculated with a LNCaP cell line (10^6 cells in BD matrigel, BD Biosciences, NJ) at both an anterior and posterior dorsal site. Tumor cells were allowed to grow until a palpable tumor appeared, approximately 50 mm^3 (normally takes 4–5 weeks) at which point animals were randomly recruited into: (1) intact mice + Western diet, and (2) intact mice + Low Carb diet groups; or animals were castrated and started taking either Western or Low Carb diet on the same day, forming (3) castrated mice + Western diet, and (4) castrated mice + Low Carb diet groups. There were 9 mice per group for each of the 4 treatment groups, 36 mice in total. All animals received standard chow (5058 Irradiated PicoLab Mouse Diet 20 (crude protein not <20%; fat not <9%, fiber not>4%, added mineral not >2.5%) until ready to switch over to one of these two isocaloric diets (3.9 kcal/g). Detailed information on the contents of these diets has been previously published [7]. Mice were fed *ad libitum* throughout the study.

Tumor size (mm^3) and body mass (g) were monitored twice weekly and a weekly serum sample was collected from the tail vein to monitor blood glucose and PSA levels. Castration resistant progression occurred approximately 2–4 weeks post-castration, indicated by a surge in the PSA levels from nadir. All animals stayed on the given diet until they reached humane endpoints, signified by the loss of ≥20% body weight, tumor burden ≥10% of the body weight and/or other signs of weaknesses. Mice from Group (1) and (2) were euthanized on Day 53, whereas Group (3) and (4) on Day 72 post-treatment.

2.3. Tumor collection and homogenization

Mice were sacrificed and tumors harvested and divided into three fractions: either frozen in liquid nitrogen and stored at −80 °C for steroid analysis; or subsequently preserved in TRIzol reagent (Invitrogen, NY) for mRNA; or preserved in 10% formalin buffer and embedded in paraffin blocks for microarray. Blood was collected by cardiac puncture to extract serum. Murine tumor tissue was homogenized using the Precellys tissue homogenizer system (Bertin Technologies, France) as per the manufacturer's protocol. Briefly, tumor tissue (150–450 mg) was homogenized in chilled double-distilled water with 1X protease inhibitor at a 1:3 (tissue:buffer) ratio using Precellys Tissue Homogenizing CKMix

(Cat. # 3961-1-009) at 6000 rpm for two cycles of 20 s each with a 15 s break.

2.4. Assays for glucose, PSA, insulin, IGF-1 and growth hormone

Blood glucose was measured using a OneTouch Ultra glucose meter and LifeScan test strips. Serum levels of PSA were evaluated using Electro-chemiluminescence total PSA immunoassay (eCobas Analyzer, Roche). Serum insulin, IGF-1, and GH levels were measured using commercial ELISA kits (Mercodia, Uppsala, Sweden; R&D Systems, MN; and Millipore, MI, respectively).

2.5. LC–MS/MS measurement of tumor steroid and serum cholesterol levels

Steroid analysis was carried out as previously described [14,30] and cholesterol methodology is similar to that of [32]. Tumor homogenates and serum were both extracted with hexane:ethyl acetate (hex:EtOAc 70:30 v/v) for steroid determination. Deuterated testosterone and DHT (d3T, d3DHT, C/D/N Isotopes) were included as internal standards (IS). Extracts were pooled, dried down (Centrivap, LabConco Inc., Kansas City, MO) and the residue derivatized with 50 mM hydroxylamine in 50% MeOH. For cholesterol analysis, serum was extracted using methanol: methyl *tert*-butyl ether (MeOH:MTBE, 20:80 v/v) with water to provide improved phase separation. Deuterated cholesterol (d6C, C/D/N Isotopes) was included as IS. Extraction was repeated with MTBE as above and residues derivatized with acetyl chloride in chloroform ($CHCl_3$). Samples were finally dried and reconstituted in MeOH: $CHCl_3$ (70:30 v/v).

Analysis was carried out with a Waters Aquity UPLC Separations Module coupled to a Waters Quattro Premier XE Mass Spectrometer. 2.1 × 100 mm and 2.1 × 50 mm, BEH 1.7 μM C18 columns were used for the steroid and cholesteryl derivatives respectively. Steroid mobile phase consisted of water (A) and 0.1% formic acid in acetonitrile (B) (gradient: 0.2 min, 20% B; 8 min, 80% B; 9–10 min, 100% B; 10.2 min, 20% B; 12 min run length). Cholesteryl ester mobile phase consisted of acetonitrile/0.1 M ammonium acetate 9/1 (A) and isopropanol (B) (gradient: 0.2 min, 25% B; 5–8 min, 70% B, 8.1 min, 25% B; 10 min run length). All data were collected in ES+ by multiple reaction monitoring (MRM) for steroids and cholesterol (as acetyl derivative), or precursor scanning (PC) for cholesteryl esters. Instrument parameters were optimized for the *m*/*z*'s and corresponding fragments of the oxime–steroids and acetyl-cholesterol monitored for each MRM with PC parameters similar the cholesterol MRM. Data processing was done with Quanlynx (Waters) and exported to Excel for normalization to weights and volumes as required. Putatative cholesteryl esters were identified based on precursor mass with calibration against cholesteryl oleate for all esters. Calibration ranges of 0.01–10 ng/ml and 0.2–10 μg/ml were used for steroids and cholesterol respectively ($R^2 > 0.98$). Recoveries and conversions to derivatized species were greater than 80% for steroids, 90% for cholesterol and 75% for cholesterol oleate.

2.6. Steroidogenic enzyme quantitation by Western blot analysis

Proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer and Western blot was performed as previously described [33]. Signals were detected and proteins levels quantified using an Odyssey Infrared Imaging system (LI-COR Biosciences) following the manufacturer's recommendations. The densitometric readings of the steroidogenic proteins were standardized using the corresponding GAPDH loading control. The fold induction or reduction of various proteins was compared to that of the Western diet group.

2.7. Expression of steroidogenic enzyme mRNA by qRT-PCR

Total RNA was extracted from tumor tissue using TRIzol reagent. Two micrograms of total RNA were reversed transcribed using random hexamers (Applied Biosystems) and MMLV (Invitrogen). Real-time PCR was performed on an ABI ViiA7 Real-Time PCR System with FastStart Universal SYBR Green Master Mix (Roche). Target gene expression was normalized to β-actin levels in respective samples as an internal standard, and the comparative cycle threshold (C_t) method was used to calculate relative quantification of target mRNAs. Each assay was carried out in triplicate. Primer sequences (5′–3′) used in this study: AKR1C3 (forward: f) tgggaggccatggagaag, (reverse: r) tttgacaccccaatggacttg; SRD5A1 (f) acgggcatcggtgcttaat, (r) ccaacagtggcataggctttc; HSD17B3 (f) tgggacagtgggcagtga, (r) cgagtacgctttcccaattcc; RDH5 (f) gcccgccagcaatgc, (r) cgcccaaagcctgagtca; HSD3B2 (f) cgggcccaactcctacaag, (r) ttttccagaggctcttcttcgt; FASN (f) cgctcggcatggctatct, (r) ctcgttgaagaacgcatcca; β-actin (f) gctcttttccagccttcctt, (r) cggatgtcaacgtcacactt.

2.8. Tissue microarray immunohistochemistry

Tumors were sectioned and stained with hematoxylin and eosin (HE) and the desired areas marked along with their corresponding paraffin blocks. The TMAs were manually constructed (Beecher Instruments, MD) by punching quadruplicate cores of 1 mm for each sample giving a total of 144 cores. All scoring was done blind with respect to treatment by LF and based on relative immunoreactive intensity on a four-point scale. However, AR was scored based on % of positive cells per core.

2.9. Statistical analyses

All data were tested for compliance with both normality (Kolmogorov test) and equal variance (Levene's test) assumptions and data were log-transformed where necessary. Changes in tumor burden, PSA, body mass and blood glucose between diets during the course of the study were assessed using repeated-measures analysis of variance. Serum and tumor concentrations were assessed using two-way ANOVA with diet and castration as fixed factors and tumor burden as a random factor. Treatment differences in immunohistochemical scores were tested using Kruskal–Wallis ANOVA for non-parametric ordinal data. Data were tested for the presence of outliers (Chauvenet's criterion) and these were removed only when it altered the significance of the analysis. Data were analyzed using Statistica 6.0 (StatSoft Inc., Tulsa, OK) and differences were significant at $P \leq 0.05$, except for Western blot and qRT-PCR data where smaller sample sizes ($N = 3, 4$) necessitated our use of an α level of $P \leq 0.1$.

3. Results

3.1. Low carbohydrate-high protein diet delays CRPC progression in LNCaP xenograft mice

To investigate how diet type impacts CRPC progression, we compared LNCaP tumor growth profiles of mice fed either a Low Carb or Western diet. No differences in LNCaP tumor progression (total tumor volume) with diet was observed for intact mice ($P = 0.471$). Importantly, however, castrated mice on the Low Carb diet exhibited a slower progression than those on the Western diet ($P = 0.017$) (Fig. 1A). Castrated mice on the Western diet resembled both groups of intact mice. In tracing serum PSA levels, a sentinel elevation, evident in castrated mice, suggested a complete progression to castration-resistance in both diet groups ($P < 0.001$; Fig. 1B). Diet did not alter serum PSA levels within either intact

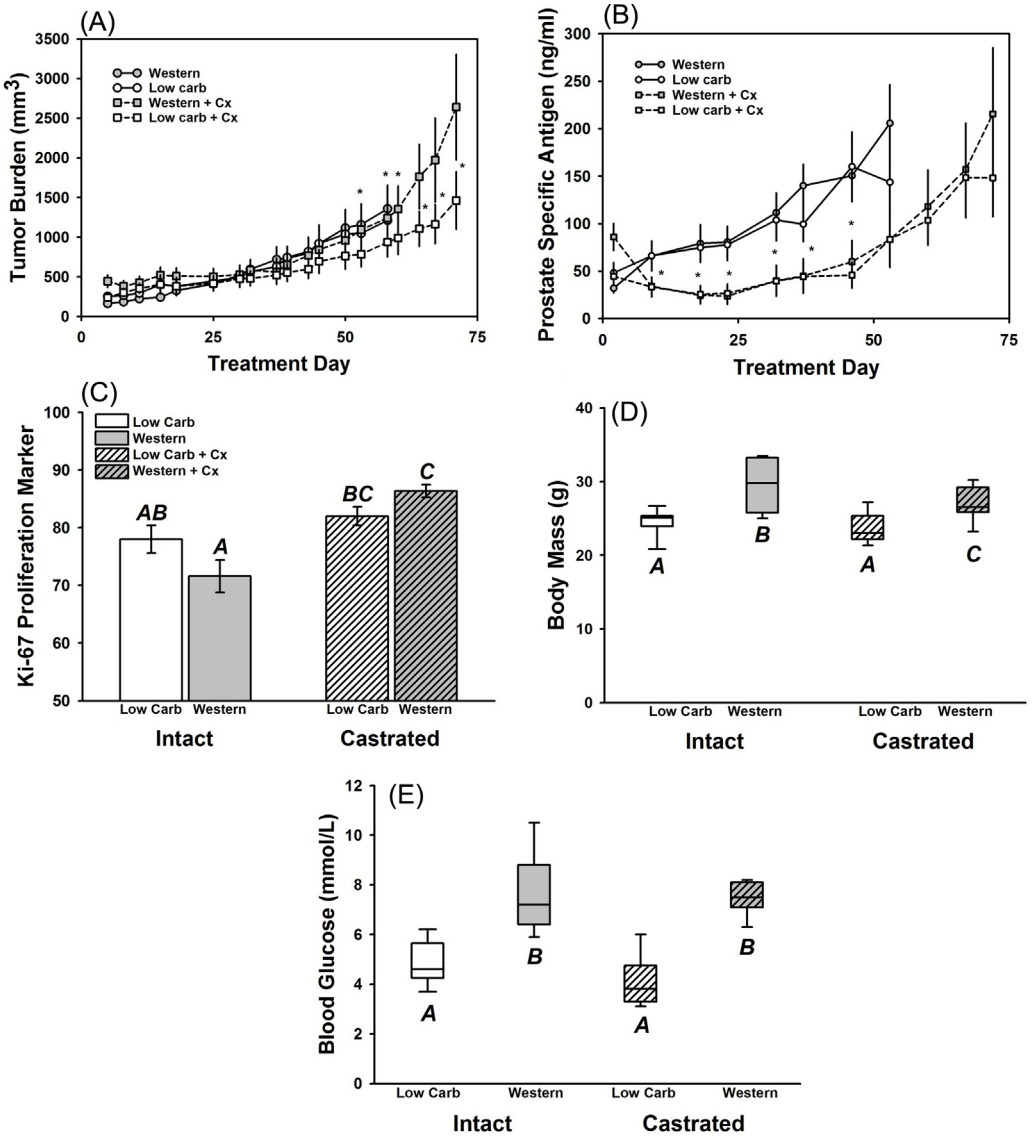

Fig. 1. CRPC tumors grow slower in mice kept on a low carbohydrate- high protein diet as compared to a Western diet. (A) Total growth of LNCaP tumors in both intact or castrated athymic "nude" mice on two diet treatments, a low carbohydrate-high protein diet (Low Carb) and a Western diet. (B) Time course changes in serum levels of prostate specific antigen in both castrated and intact mice held on two diets. All data are shown as means ± standard errors. * denotes significant differences at $P < 0.05$. (C) Comparison of intratumoral levels of the cell proliferation biomarker Ki-67 in tumors grown on mice held on two different diets. Data are shown as means ± standard errors and different letters indicate significant differences at $P < 0.05$. Boxplots indicating means (center lines), 95% confidence intervals (boxes) and ranges (bars) in body mass (D) and blood glucose levels (E) in both intact and castrated mice held on two different diets.

mice ($P = 0.998$) or castrated mice ($P = 0.999$). Expression of Ki-67, a cell proliferation biomarker, was higher in castrated mice on the Western diet than their intact counterparts ($P = 0.004$; Fig. 1C), but no such difference was observed for the Low Carb groups ($P = 0.451$; Fig. 1C).

Mice did not differ in body mass at the onset of the study ($P = 0.908$), but as the study progressed, intact mice kept on the Western diet rapidly gained weight and by the end of the study were about 15% heavier than the other groups ($P < 0.001$; Fig. 1D). This mass-promoting effect of the Western diet was more apparent in intact mice than in castrated mice, suggesting a role for systemic androgens in weight gain. This pattern in body mass was mirrored by blood glucose levels, with those on the Western diet having higher levels than those of the Low Carb diet, regardless of effects of castration ($P < 0.001$; Fig. 1E).

3.2. Low carbohydrate-high protein diet reduces DHT content in CRPC mice

Differences in tumor progression with diet type (Fig. 1A) may be explained by variation in local androgen synthesis (namely DHT) which could reactivate AR signaling [9–10]. We therefore measured the levels of several steroids using LC–MS/MS (Fig. 2A) in LNCaP tumor samples to determine whether decreased tumor growth in the Low Carb group was associated with lower local T and/or DHT concentrations. Both castration and diet altered steroid content in tumor tissues (Fig. 2B). As expected, castration drastically lowered T levels in both castrated groups ($P < 0.001$; Fig. 2B) but no differences between diets were observed (intact: $P = 0.907$; castrated: $P = 0.902$). Functionally, T is converted within tumor cells to DHT, which then binds to the AR with higher affinity [34]. Intratumoral DHT content was similar between intact mice on

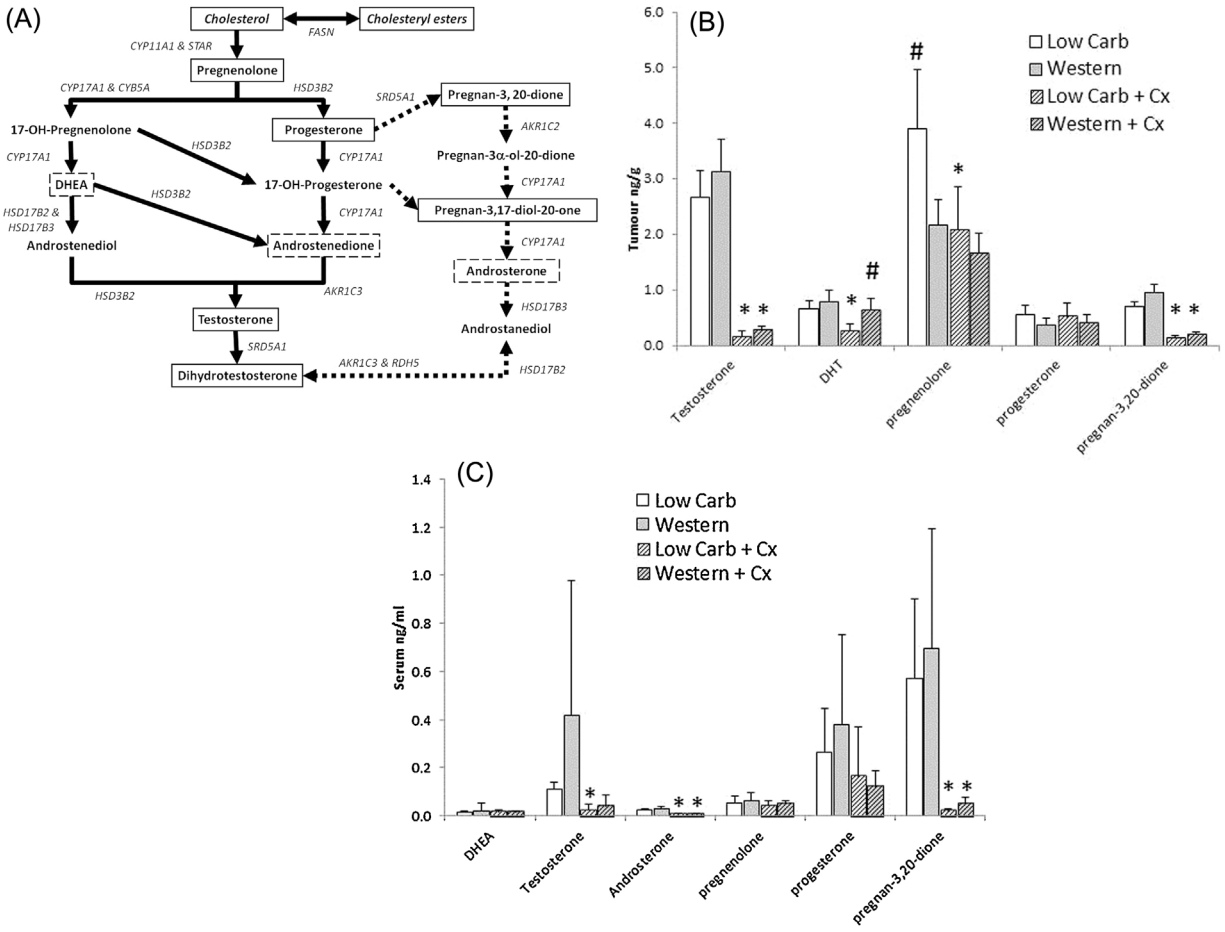

Fig. 2. Lower DHT concentrations in LNCaP tumors from mice fed a low carbohydrate- high-protein diet. (A) Steroidogenesis pathway from cholesterol (and its esters) to dihydrotestosterone through both classical (solid arrows) and backdoor (dashed arrows) pathways. Cholesterols and steroids (in bold) measured using LC–MS/MS are indicated in boxes (dashed boxes indicate those below level of quantification: see methods). (B) Concentrations of steroids measured within tumor homogenates isolated from intact and castrated mice fed one of either a low carbohydrate-high protein (Low Carb) or a higher carbohydrate-lower protein (Western) diet. All data are shown as means ± standard errors. * indicates a significant difference with castration, and # indicates significant differences between diets, both at $P \leq 0.05$. (C) Concentrations of steroids measured in serum from the same intact and castrated mice as in B along with similar figure annotation.

a Western diet or Low Carb diet ($P = 0.466$), but importantly, intratumoral DHT concentrations were significantly decreased in CRPC-bearing mice on the Low Carb diet ($P = 0.036$). The DHT concentration with the Low Carb diet was about 58% that of the CRPC-bearing mice on the Western diet, and this low DHT concentration (0.267 ± 0.080 ng/g of tumor tissue) should partly suppress the AR signaling reactivation associated with CRPC [34,35]. The comparable amounts of DHT in the tumors from the CRPC-bearing mice on the Western diet and the intact mice on either diet suggest a similar level of AR activation.

Interestingly, tumors from intact mice on a Low Carb diet had a 2-fold higher pregnenolone content than those from intact mice on the Western diet ($P = 0.010$; Fig. 2B) while within CRPC tumors, there was no significant difference (intact: $P = 0.082$; castrated: $P = 0.514$). Pregnenolone is the first steroid produced from cholesterol as part of the predominant steroidogenesis pathway (Fig. 2A). The higher pregnenolone content in the intact + Low Carb diet group can be interpreted in several ways including: increased cholesterol uptake and subsequent conversion to pregnenolone and an accumulation resulting from lowered steroid synthesis in intact mice with a Low Carb diet. Neither castration nor diet altered progesterone (P_4) concentrations ($P = 0.610$), suggesting that increased conversion of pregnenolone to P_4 is unlikely to explain the lower pregnenolone levels in the Western diet fed intact mice, unless P_4 is then rapidly metabolized into androgens.

Three additional steroid intermediates were also detectable in almost all samples. In spite of their low concentration (below our acceptable quantification limits for LC–MS/MS; 5–10 times background or ±20% accuracy deviation) there did appear to be a downward trend for the castrated groups. Androstenedione, an intermediate metabolite between P_4 and T, was detectable in 32 of 36 samples, and its concentration in the intact + Western diet group was about 3 times higher than the intact + Low Carb diet group, and about 4 times higher than either castrated group (data not shown). While these measurements are too low to definitively profile this part of the pathway, the multiple fold differences do suggest the possibility that a Western diet may promote increased DHT synthesis through this route. Similarly, the pathway involving conversion of pregnenolone to dehydroepiandrosterone (DHEA) could promote T synthesis; tumors from both the intact and castrated Low Carb diet groups had twice as much DHEA (detectable in 34 of 36 samples) as those from Western diet groups (data not shown). Thus, diet may alter the predominant pathway for T synthesis within tumor cells, with the Low Carb diet suppressing DHT synthesis through the direct P_4 to T pathway.

We also evaluated DHT synthesis *via* the alternate "backdoor" pathway (Fig. 2A), described by Penning [36,37] and Auchus [36,37], by measuring concentrations of two intermediate metabolites. Specifically, we measured the concentration of pregnane 3,20-dione, a P_4 metabolite, and found that castration

reduced its level significantly regardless of diet treatment ($P < 0.001$; Fig. 2B). This steroid is subsequently converted to androsterone, which was also detectable in all samples but below the acceptable limit of quantification. Castration reduced levels of this weak androgen were approximately 4× lower in castrate tumors than in the intact mice groups (data not shown), consistent with our pregnane-3,20-dione results. Serum levels were also evaluated and while differences between tumor and serum were evident, overall trends were quite similar (Fig. 2C). Androsterone was significantly reduced in the castrated groups; no differences were observed for DHEA, and androstenedione was not detected. Testosterone and 5-pregnan-3,30-dione behaved as in tumors, whereas the Low Carb effect on pregnenolone was not evident and a possible reduction in progesterone may have occurred. Thus castration, but not diet, may have a greater influence in suppressing DHT production through this backdoor pathway.

3.3. Low carbohydrate-high protein diet reduces steroids availability in CRPC mice

Since DHT synthesis occurs through several pathways (Fig. 2A), the effects of diet on steroidogenic enzymes was investigated using both Western blotting and quantitative real-time PCR. Diet regime had little effect on LNCaP tumor protein levels for most enzymes (Fig. 3A; all $P \geq 0.205$). However, tumors isolated from intact mice on the Low Carb diet had higher AKR1C3 protein levels and lower HSD17B2 protein levels than intact mice on the Western diet (ARK1C3: $P = 0.074$; HSD17B2: $P = 0.091$, with $\alpha = 0.1$; Fig. 3A). AKR1C3 catalyzes the synthesis of T from androstenedione (Fig. 2A), suggesting intact + Low Carb mice have a heightened capacity to produce T within the tumor. Interestingly, lower levels of HSD17B2 in intact + Low Carb mice, possibly indicates a decreased utilization of the backdoor pathway to synthesize DHT. In contrast, CRPC tumors from mice on Low Carb diets had higher concentrations of both HSD17B2 ($P = 0.016$; Fig. 3A) and SRD5A1 ($P = 0.058$ with $\alpha = 0.1$; Fig. 3A) enzymes. This suggests a possible up-regulation of the backdoor pathway and DHT production in the Low Carb diet group, where in fact DHT concentrations were actually lowest. These observations may point to a sensitization or compensatory effect aimed at maximizing conversion of substrate to DHT to maintain the CRPC phenotype. Expression of tumor AKR1C3 mRNA increased with castration, but this increase was significant only in mice on the Low Carb diet ($P = 0.047$; Fig. 3B) and was greater in intact mice on the Western diet compared to the Low Carb diet ($P = 0.071$ with $\alpha = 0.1$; Fig. 3B). For intact mice, expression of tumor HSD17B3 mRNA was also observed with over 2-fold higher levels in Western-fed mice compared to Low Carb mice ($P = 0.057$); however, the opposite pattern was observed in CRPC-bearing mice, where levels were higher (almost 4-fold) for the Low Carb group ($P = 0.038$). No treatment effects on expression of RDH5, HSD3B2 or SRD5A1 mRNA were observed (Fig. 3B; all $P \geq 0.414$). CRPC tumors had

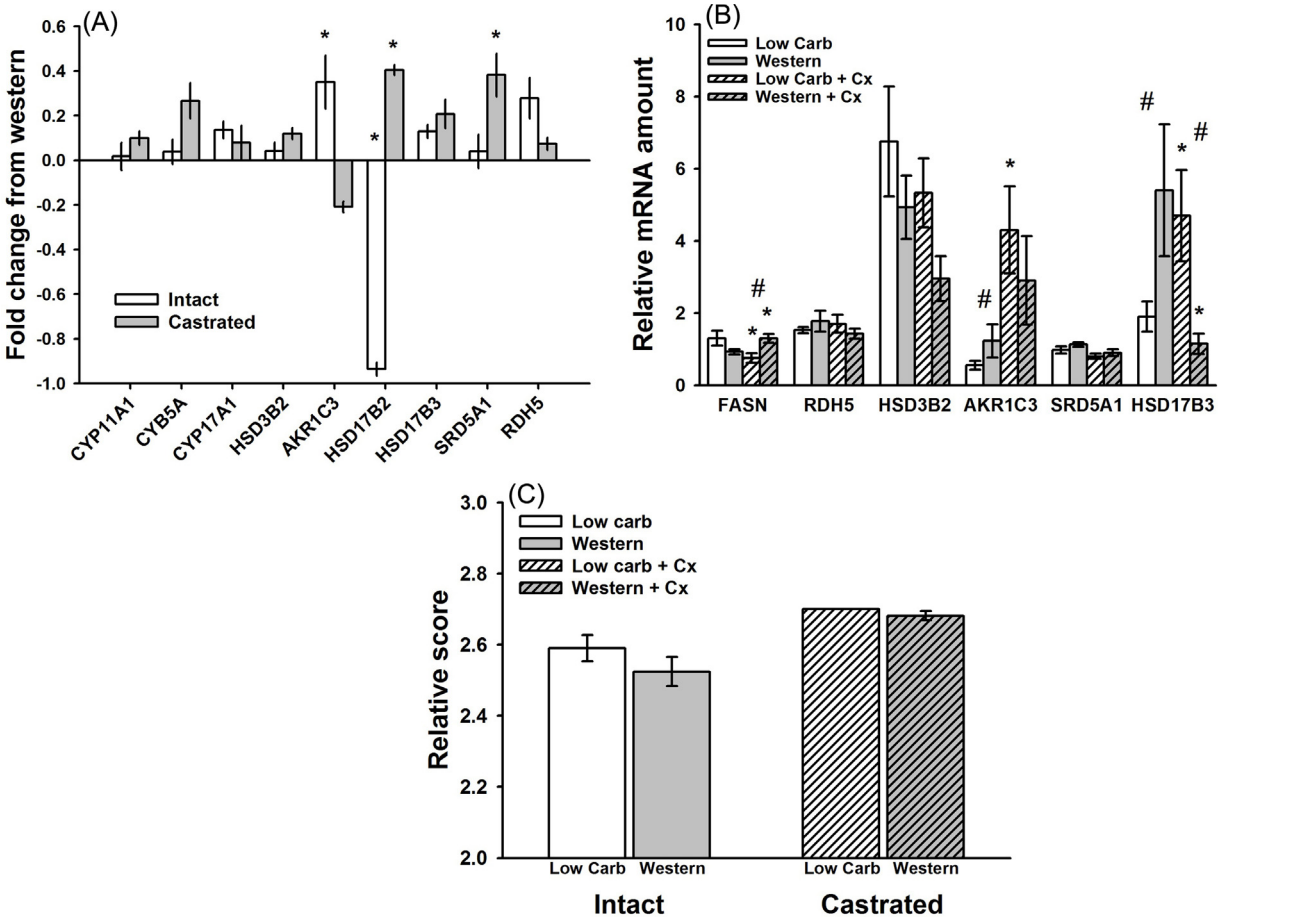

Fig. 3. Compensatory up-regulation of DHT synthesis through a backdoor production pathway in CRPC tumors from mice fed a low carbohydrate- high-protein diet. (A) Protein content of steroidogenic enzymes from LNCaP tumor homogenates obtained from mice fed a either a low carbohydrate-high protein (Low Carb) or a higher carbohydrate-lower protein (Western) diet. Bars indicate differences between the Low Carb groups relative to the similar mice held on Western diet. * indicates a significant difference between diets. (B) Expression of steroidogenic enzyme mRNA (normalized to levels of β-actin) in both intact and castrated mice on two different diets. * indicates a significant difference with castration, and # indicates significant differences between diets, both at $P \leq 0.05$. (C) No significant effect of castration or diet on intratumoral androgen receptor levels as determined by scoring of immunoreactivity in tissue microarray. All data are shown as means ± standard errors.

greater AR immunoreactivity than tumors collected from intact mice ($P = 0.003$); however, this was not influenced by diet ($P = 0.28$; Fig. 3C). These data suggest that castration induces the up-regulation of local T synthesis through the classical pathway and that this is more prevalent in mice on the Low Carb diet, since tumors in these mice were likely to be more depleted of steroid precursor substrates than tumors in mice on the Western diet.

3.4. Low carbohydrate-high protein diet decreases cholesterol availability for steroidogenesis during CPRC

The onset of CRPC is associated with shifts in cholesterol processing (uptake, synthesis, metabolism, secretion) within LNCaP cells that promote a local accumulation of cholesteryl esters, which can be cleaved to produce steroidogenic substrates [20]. We examined diet effects on serum concentrations of free cholesterol and its cholesteryl esters to assess their availability as substrates for steroidogenesis in CRPC. Both castration ($P = 0.016$) and diet ($P = 0.009$) had significant effects on free cholesterol levels (Fig. 4A). As expected, intact mice on the Western diet had higher free cholesterol concentrations than intact mice on the Low Carb diet ($P = 0.008$). Castration increased free cholesterol levels in the Low Carb diet groups ($P = 0.014$), but not in the Western diet groups. Serum concentrations of several cholesteryl esters were significantly higher in intact mice on the Western diet than the Low Carb diet (Fig. 4A), including those conjugated to oleic ($P = 0.013$), linoleic ($P = 0.028$) and arachidonic ($P \leq 0.001$) acids. In contrast, the opposite relationship was observed in cholesteryl esters composed of eicosapentanoic and docosahexanoic acids (both $P \leq 0.001$; Fig. 4A). This is likely due to the slightly higher levels of omega-3 fatty acids (FAs) in the Low Carb chow (0.58% of total calories *versus* 0.14% in the Western chow). Castration significantly increased cholesteryl esters conjugated to oleic ($P = 0.043$) and arachidonic acids ($P = 0.048$) but only in the Western fed animals (Fig. 4A). Among castrated mice, serum levels of cholesteryl esters were higher for the Western diet group than the Low Carb diet group for oleic ($P = 0.035$) and arachidonic ($P \leq 0.001$) acids, but the Western diet group had lower serum levels of eicosapentanoic and docosahexanoic acids (both $P \leq 0.001$; Fig. 4A), likely because of the higher omega-3 FA levels in the Low Carb chow.

Uptake of circulating cholesterol into tumor cells is mediated by receptor transport of both high-density and low-density lipoproteins (HDL and LDL, respectively) [38,39]. Previous reports have documented increased expression of LDL receptor (LDL-R) with prostate cancer progression, suggesting increased cholesterol influx into tumor cells [40]. HDL trafficking is mediated by the scavenger receptor class B type 1 (SR-B1), which is expressed in LNCaP cells [19,20,41] and considered to be the primary pathway of cholesterol delivery to steroidogenic cells [42,43]. After outlier

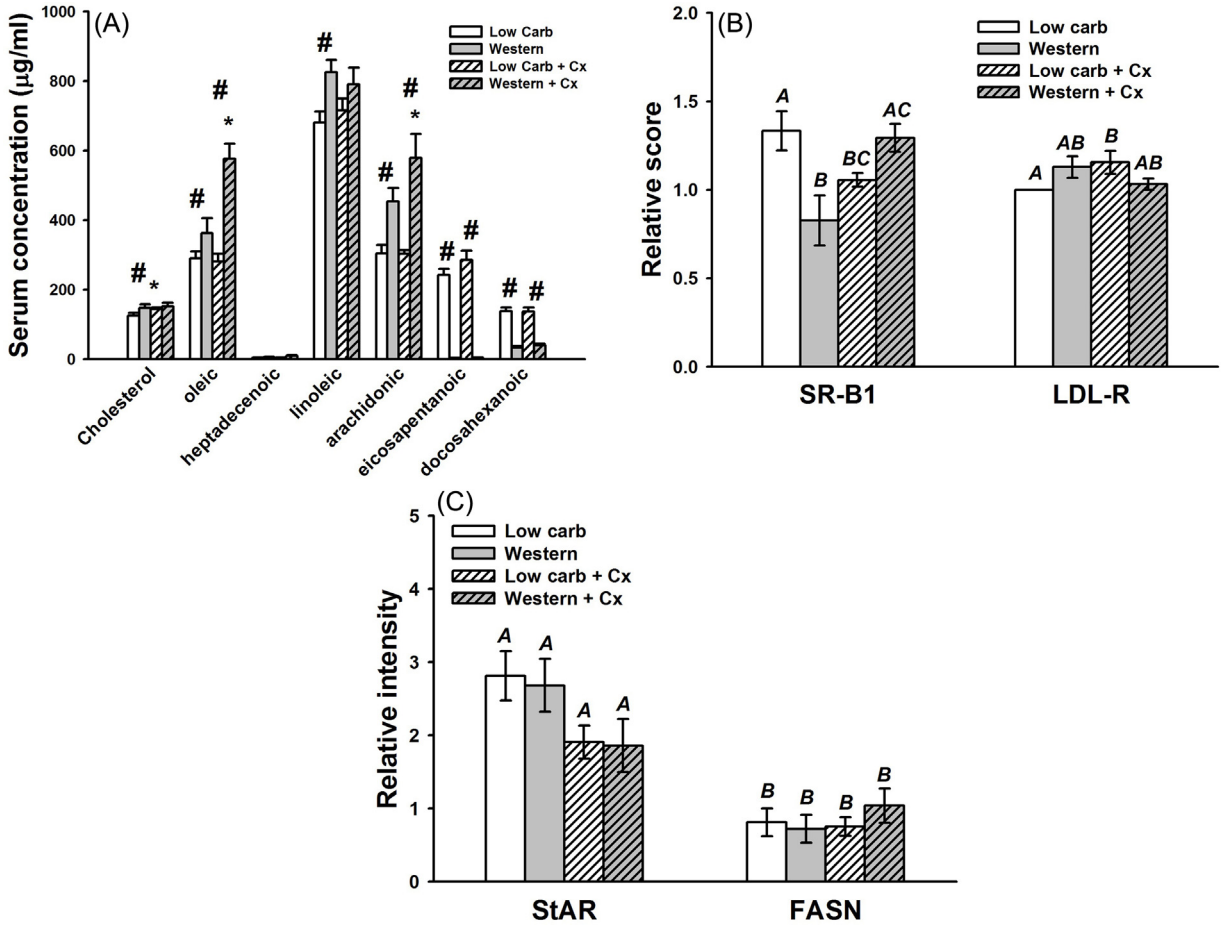

Fig. 4. Decreased cholesterol availability for CRPC tumors on mice fed a low carbohydrate-high-protein diet. (A) Serum concentrations of cholesterol and cholesteryl esters formed through its conjugation with various fatty acids in both intact and castrated mice fed either a low carbohydrate-high protein (Low Carb) or a higher carbohydrate-lower protein (Western) diet. * indicates a significant difference with castration, and # indicates significant differences between diets, both at $P \leq 0.05$. (B) Relative immunoreactivities in tumor samples for the scavenger receptor B1 (SR-B1) and the low-density lipoprotein receptor (LDL-R) indicative of the cellular uptake of high and low-density lipoprotein, respectively. Different letters indicate significant differences at $P < 0.05$. (C) Effect of castration and diet on tumor protein content of steroidogenic acute regulatory protein (StAR) and fatty acid synthase (FASN) determined by Western blotting. All data are shown as means ± standard errors.

removal, both SR-B1 and LDL-R immunoreactivity in LNCaP tumors differed between groups (SR-B1: $P = 0.047$; LDL-R: $P = 0.031$; Fig. 4B). For SR-B1, immunoreactivity was lower for intact mice on the Western diet than the Low Carb diet ($P = 0.038$; Fig. 4B). Castration significantly decreased SR-B1 immunoreactivity in the Low Carb diet groups ($P = 0.029$; Fig. 4B) but increased it in the Western diet groups ($P = 0.019$; Fig. 4B). In contrast, LDL-R immunoreactivity in tumors increased with castration in the Low Carb diet groups ($P = 0.029$; Fig. 4B) to levels greater than the castrated Western diet group ($P = 0.024$; Fig. 4B).

Transportation of cholesterol into mitochondria is mediated by the steroidogenic acute regulatory protein (StAR), considered the rate-limiting step in steroid synthesis [15,42,44]. No differences in intratumoral StAR protein content were detected amongst treatments ($P = 0.576$; Fig. 4C). In addition to cholesterol availability, progression to CRPC is mediated by the up-regulation of enzymes that regulate endogenous fatty acid synthesis, including fatty acid synthase (FASN) [24,45]. Intratumoral FASN protein content did not differ with diet or castration ($P = 0.853$; Fig. 4C). FASN mRNA expression also did not differ with diet in tumors from intact mice ($P = 0.085$; Fig. 3B), and castration increased and decreased tumor FASN expression in Low Carb and Western diet groups, respectively (Low Carb: $P = 0.017$; Western: $P = 0.045$; Fig. 3B). Furthermore, castrated Low Carb mice had lower tumor FASN mRNA expression than counterparts on the Western diet ($P = 0.017$; Fig. 3B).

3.5. Progression of CRPC tumors in LNCaP xenografts is not-related to either insulin or GH/IGF-1 signaling pathways

Since the Low Carb diet decreased blood glucose levels, we predicted that it would also reduce serum insulin levels. Indeed, we found that intact Low Carb mice had a 34% lower insulin concentrations than intact mice on the Western diet ($P = 0.057$; Fig. 5A). Surprisingly, however, no difference in insulin levels was apparent in castrated mice on the different diets (Fig. 5A). We therefore explored whether diet could alter the insulin receptor (IR). Tumors from intact mice on the Western diet had higher IR immunoreactivity than Low Carb counterparts ($P = 0.038$; Fig. 5B) or castrated mice (Low Carb: $P = 0.036$; Western: $P = 0.001$; Fig. 5B). Thus, a Western diet increased sensitivity to already higher levels of insulin, but castration attenuated this response. Insulin signaling *via* IR is facilitated by adapter proteins, including the insulin receptor substrate type 2 (IRS-2), which recruits effectors during insulin stimulation [46]. Positive associations between IRS-2, cancer cell proliferation and glycolysis have been previously reported [47–49]. However, tumor IRS-2 immunoreactivity did not differ between diets or with castration (all $P > 0.683$; Fig. 5B).

Another potential pathway involves insulin-like growth factor 1 (IGF-1) and its primary secretagogue, growth hormone (GH). Many tumor types, including prostate tumors, show increased expression of IGF-1 receptor (IGF-1R) [50,51], which may stimulate

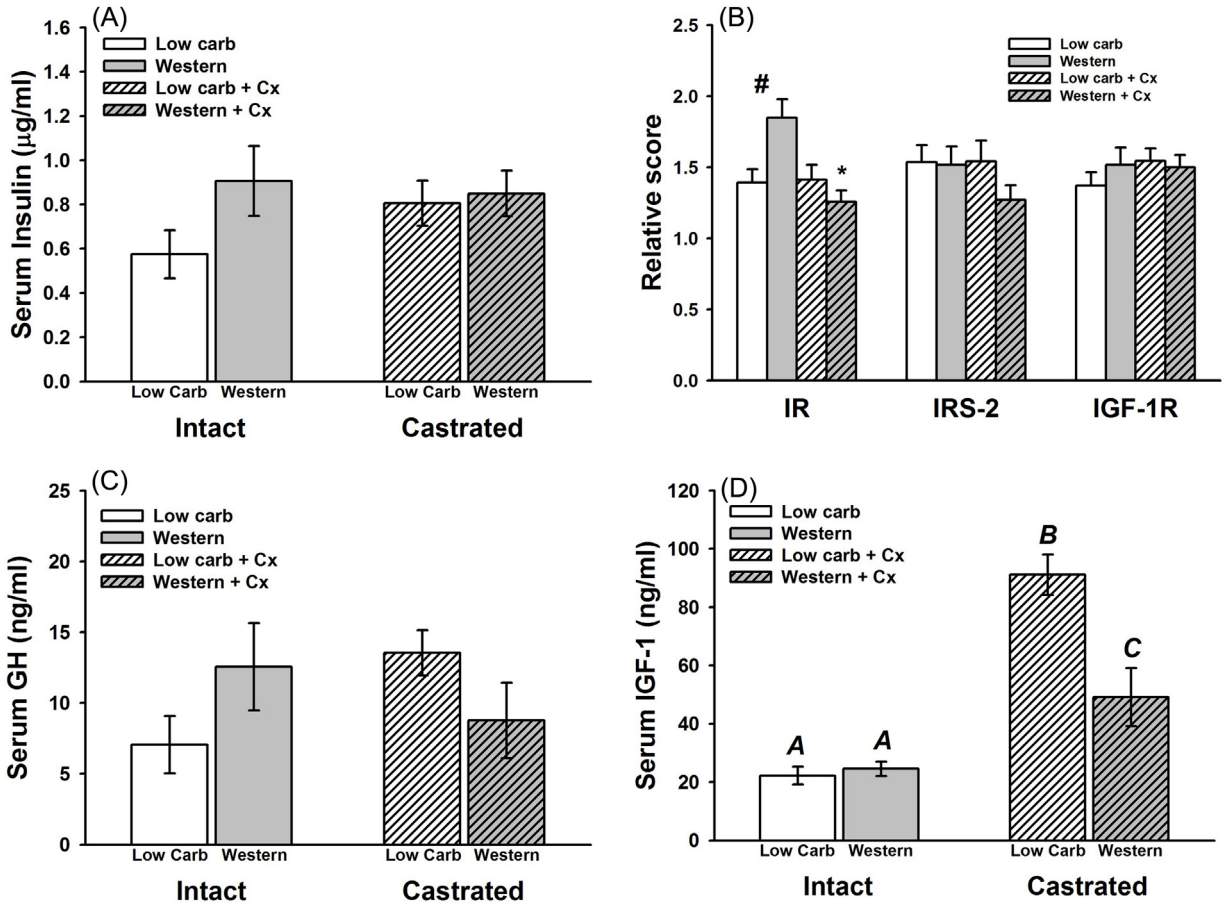

Fig. 5. No evidence for an effect of diet on insulin, IGF-1 or GH signaling in CRPC tumors. (A) Serum insulin levels in intact and castrated mice fed either a low carbohydrate-high protein (Low Carb) or a higher carbohydrate-lower protein (Western) diet. (B) Relative immunoreactivities in tumor samples for the insulin receptor (IR), the insulin receptor substrate (IRS-2), and the insulin-like growth factor receptor (IGF-1R) for intact and castrated mice on one of two diets. * indicates a significant difference with castration, and # indicates significant differences between diets. Serum concentrations of (C) growth hormone (GH) and (D) insulin-like growth factor-1 (IGF-1) in intact and castrated mice fed either a Low Carb or Western diet. Different letters indicate significant differences at $P < 0.05$ and all data are shown as means ± standard errors.

mitogenesis and promote cell survival [52] by complementing insulin-induced effects [52,53]. While there was a trend for higher and lower serum GH levels in the Western diet group in intact and castrated mice respectively, there was no significant effect of treatment ($P = 0.069$; Fig. 5C). In contrast, serum IGF-1 concentrations did not differ between intact mice on the two diets ($P = 0.597$), but increased in response to castration in both diet groups (Western: $P = 0.054$; Low Carb: $P < 0.001$; Fig. 5D). Surprisingly, in castrated mice, serum IGF-1 levels were higher in the Low Carb diet group than the Western diet group ($P < 0.001$; Fig. 5D). However, no differences in intratumoral IGF-1R were observed across treatments ($P = 0.880$; Fig. 5B).

4. Discussion

There is a wealth of clinical data suggesting that obesity negatively impacts prostate cancer prognosis and treatment outcomes [54,55]. Obesity is associated with metabolic syndrome, which has become epidemic in societies that have adopted a high-carbohydrate, high-fat "Western" diet. Metabolic syndrome is characterized by high levels of blood insulin and glucose, which can promote aggressive tumor growth in animal models [7,29]. Castration/ADT can also induce metabolic syndrome in patients, which includes both elevated fasting serum insulin and glucose levels. This effect of ADT may be enhanced by high-carbohydrate, high-fat diets [28].

The slow-growing nature of prostate cancer provides an ideal model in which to test the effect of diet on cancer progression. The current study demonstrates that, as predicted, tumor growth in castrated mice is significantly reduced by a Low Carb (high protein) diet when compared with a typical Western diet. Interestingly, this effect was demonstrated only in castration-resistant xenograft bearing mice. Tumor growth in intact mice was not affected significantly by the diets tested. Multiple mechanisms are known to be impacted by the availability of a high carbohydrate load. Previous research demonstrated increased *de novo* steroidogenesis in LNCaP cells deprived of androgens [30]. Here, we shed light on a specific *de novo* steroid synthesis mechanism, previously observed in CRPC progression, which may be important in contributing to our study outcomes.

Intratumoral steroid levels were impacted significantly by both castration and diet. Not surprisingly, castration caused levels of T, DHT and pregnan-3,20-dione to plummet. However, these steroids were still detectable even in the absence of testicular-derived circulating androgens, which supports the hypothesis that AR-mediated tumor growth relies in part upon local *de novo* androgen synthesis [14,24]. The selective nature of the effects of the Low Carb diet in impacting only the castrated mice with CRPC tumors led us to explore local steroid synthesis as a main driver mediating tumor growth inhibition. Both steroidogenic enzyme synthesis machinery and production of androgenic steroids were significantly higher in xenograft tissues of mice fed a Western diet rather than a Low Carb diet. Of note, levels of DHT in particular were higher in tumor tissues of mice in the Western diet group. The supply of DHT required for reactivation of AR-mediated castration-resistant tumor growth is therefore likely to be responsible for the more aggressive tumor growth observed in the Western diet group.

It has been previously shown that the AKR1C3 enzyme of the steroid pathway is up-regulated in response to castration in clinical patient samples [56]. AKR1C3 mediates the pro-androgenic conversion of androstanediol and androstenedione to T [30,56]. We confirm that this up-regulation also occurs in the LNCaP model (Fig. 3B), which recapitulates prostate cancer and progression to CRPC upon castration-mediated hormone withdrawal. While no statistically significant difference was found in protein levels of AKR1C3 between the two diet groups, we showed that AKR1C3 mRNA levels are higher in the intact Western diet group, as well as both castrated groups, compared with the intact Low Carb diet group. In addition, we observe a significant increase in HSD17B2 enzyme protein levels in the intact Western diet group, which is not mirrored in the castrated animals where the opposite trend was found (but not statistically significant). The HSD17B2 enzyme converts DHT back to androstanediol as part of a 'backdoor' pathway while also being responsible for the conversion of DHEA to androstenediol occurring further upstream in the steroidogenesis pathway (Fig. 2B). These steroidogenic properties of HSD17B2 would seemingly counteract each other with respect to T and DHT synthesis, and opposing trends in steroid profiling for intact and castrated groups fed a Western *versus* Low Carb diet are therefore not completely unexpected.

While blood glucose levels were significantly lower in mice on a Low Carb diet, neither serum insulin, intratumoral insulin receptor (IR) or serum growth hormone (GH) levels were impacted significantly by diet. A recent *in vitro* study [30] suggested that steroidogenic enzyme activity and steroid production are regulated by insulin and related signaling components. However, here we are unable to confirm this regulatory process for steroidogenesis *in vivo*. Our current data confirms a recent study reported for the LAPC-4 tumor model, in which there was also no correlation between insulin levels and the effect of a Low Carb diet on tumor growth (54). Similarly, although we do see an anticipated increase in serum IGF-1 upon castration (55), we observe a significant drop in serum IGF-1 as a result of the Western diet which does not correlate with the increased tumor growth in this group. As expected, body weight profiles were strongly affected by diet, with a significant increase in body mass in animals on the Western diet *versus* the Low Carb diet.

FASN is involved in the manufacture of long-chain fatty acids and, alongside cholesterol, FASN is thought to contribute substrates required for steroidogenesis. Interestingly we saw no changes in intratumoral FASN gene expression regardless of castration status or diet, but serum cholesterol levels were strongly predictive of tumor growth and affected very strongly by diet. The Low Carb diet resulted in lower levels of several important serum cholesteryl esters. This is likely attributable to the compositional differences between the diets with respect to omega-3 fatty acids. Interestingly, a difference in free cholesterol only existed for the intact mice on the Low Carb diet, which had lower levels than the other groups, including the castrated mice on the Low Carb diet. The fact that the castrated groups were both high in serum cholesteryl esters (alongside the intact Western diet group) is in accordance with previous literature that reports an increase in cellular cholesteryl esters following castration [24,57]. Absence of any further elevation of serum cholesteryl esters in the castrated group fed the Western diet suggests that the capacity of the serum to carry cholesteryl esters may be saturable. In keeping with this, we observe significant group differences in intratumoral levels of the cholesterol transporter SR-B1. This ATP binding cassette (ABCA-1) transporter facilitates the cellular uptake and efflux of serum cholesterol, as mediated both to and from serum high density lipoprotein (HDL) [39,58]. This is the major pathway thought to be responsible for enhanced cholesterol uptake into steroidogenic cells, including prostate cancer cells [20]. The Western diet fed intact mice had a lower level of SR-B1, which was statistically significant as compared with the intact Low Carb diet fed mice or the castrated groups. In an environment where there is no required capacity of tumor tissue to be steroidogenic, such as in intact mice, one could postulate that less SR-B1 would be required to regulate cholesterol uptake for steroidogenesis. In contrast, once mice are castrated, the tumors acquire the resistance phenotype associated with steroidogenesis using cholesterol as a precursor substrate, hence SR-B1 levels would

be expected to increase (as observed). Interestingly we see higher, castrate equivalent levels of SR-B1 for tumor tissues derived from the Low Carb diet fed intact animals. This may suggest an increased capacity for intratumoral uptake of cholesterol for growth, beyond the levels required for steroidogenesis, when present in a cholesterol depleted environment. LDL-receptor (LDL-r) also goes up in the Low Carb diet treated groups upon castration. LDL cholesterol uptake is mediated by the steroid regulatory enzyme binding protein (SREBP)-regulated LDL-r, the expression of which increases during clinical CaP progression [40]. The LDL-r increase in the Low Carb but not the Western diet fed castrated mice suggests that enhanced LDL-r is required in an environment depleted of serum cholesterol, to facilitate uptake.

In summary, we have demonstrated that in the castrated environment, a Low Carb diet has the potential to slow prostate tumor growth. Our results suggest that this phenomenon could be occurring as a result of the impact of a Low Carb diet on serum cholesteryl esters, which are the precursor substrates required for intratumoral steroidogenesis. We have demonstrated that tumor steroid profiles are altered as a result of diet, as are the associated steroidogenic enzymes, evoking enhanced DHT production as part of the previously well-characterized *de novo* steroidogenesis resistance process [14,21,30].

Conflict of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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