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TITLE: Vitamin D Deficiency Leads to Increased Intraprostatic Hormones in African American Men

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14. ABSTRACT Our most significant finding for Y2 of this project is the profound defect in prostate differentiation observed in the organoids lacking Megalin, which supports its role in hormone import. We have also focused in HSD17B7 as a key gene differentially expressed by African Ancestry. HSD17B7 is a key enzyme for estrogen metabolism and we found that it is higher in AA prostate stroma on a TMA. Further, HSD17B7 expression is increased by estrogen and androgen in patient-derived stromal cells, validating the microarray data.					
15. SUBJECT TERMS prostate cancer, vitamin D, androgen, testosterone, megalin, Lrp2					
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1. INTRODUCTION

This proposal is built around our hypothesis that intra-prostatic androgens and estrogen may be the link between two undisputable disparities in African American (AA) men; vitamin D deficiency and prostate cancer (PCa). We propose that the molecular response of prostate tissues to low vitamin D status leads to increased intracellular import of not only vitamin D, but also androgens and estrogens. In other words, intra-prostatic levels of estrogens and androgens may be higher in AA men because of a molecular “door” that was opened for vitamin D. This study may also shed light on inconsistent findings regarding circulating levels of hormones and PCa risk. Hormones, including vitamin D, androgens and estrogen, are bound to serum globulins with only 1-3% existing as free hormones in the circulation. The “Free Hormone Hypothesis” is commonly accepted and assumes that only unbound free hormones are accessible to tissues via diffusion, thus tissue hormone levels mirror circulating levels in the blood. Our recent findings in AA men challenge the Free Hormone Hypothesis as we have shown that prostate tissues express functional megalin protein, a membrane endocytic receptor, to internalize globulin-bound hormones. Megalin function is well-described in the kidney, but there is a paucity of studies on prostatic megalin. The Free Hormone Hypothesis excludes the presence of extra-renal megalin entirely. Both vitamin D binding protein (DBP) and sex hormone binding globulin (SHBG), which binds androgens and estrogens, bind to megalin for endocytosis. We observed that in AA men only, the expression of prostatic megalin increased with vitamin D deficiency and increased with the percentage of West African Ancestry.

We propose that long term vitamin D deficiency in AA men leads to a compensatory increase in megalin to maintain tissue levels when the serum is deficient. In turn, high megalin would also import more androgens and estrogens, thus driving hormonal carcinogenesis.

Hypothesis: Low vitamin D status in African American men drives increased androgen and estrogen import into the prostate via the membrane endocytic receptor megalin. The objective is to both determine clinical significance and to delve into the mechanisms that regulate of hormone import. The effect of vitamin D deficiency on the serum and intra-prostatic distribution of androgens/estrogens will be examined in Aim 1. The results of Aim 1 will define the relationships between the hormones through correlative data. Whereas the experiments in Aim 2 will demonstrate that megalin as the mechanism of endocytic import for hormones using an *in vitro* model. Lastly, the third Aim will examine if pathways that are active in patient tissues are regulated by intra-prostatic hormone levels. Importantly, all of these experiments utilize patient specimens and patient-derived cells from benign areas of the prostate, which is critical to assess activities of vitamin D in regards to prostate cancer risk.

2. KEYWORDS: prostate cancer, vitamin D, androgen, testosterone, megalin, Lrp2

3. ACCOMPLISHMENTS:

What were the major goals of the project? What was accomplished under these goals?

		UIC (Nonn) (Prins) (Chen) (Baumann)	COH (Kittles)	UPenn (Penning)
SPECIFIC AIM 1: To determine relationship between intra-prostatic concentrations of androgens and estrogens with vitamin D status in African American men.	Timeline (months)			
Major Task 1: Measure and quantify hormones in patient serum and frozen prostate tissue in Cohort 1	1-36			
Subtask 1: Procure de-identified tissue for LC-MS/MS optimization and send to Penning Lab	1-2	Completed		
Subtask 2: Optimize LC-MS/MS method	2-6			Completed
Major Task 2: Measure and quantify hormones in patient serum and frozen prostate tissue in Cohort 2				

Subtask 1: Procure all patient tissues and sera for Cohort 2 from UIC Biorepository and PCBN and send to Penning Lab	2-9	N=209 samples collected		N=63 sample awaiting analysis
Subtask 2: Perform LC-MS/MS on Cohort 2 patient samples	12-24			In progress
Major Task 3: Determine West African Ancestry estimates patients in Cohort 2				
Subtask 1: Procure all buffy coat for Cohort 2 from UIC Biorepository and PCBN and send to Kittles Lab	2-9	Completed		
Subtask 2: DNA isolation and SNP ancestry analysis of Cohort 2	9-24	DNA isolated	Not started	
Subtask 3: SNP analysis of vitamin D-related polymorphisms in Cohort 2	9-24		Not started	
Major Task 4: Statistical analysis of the data				
Subtask 1: Collect and organize data from Cohort 1 and meet with Dr. Yi-fan Cheng in UIC Analysis core	9-12	Completed		
Subtask 2: Analysis of Cohort 1 by Dr. Yi-Fan Chen	12-18	Completed		
Subtask 3: Collect and organize Cohort 2 data from Penning and Kittles labs and meet with Dr. Yi-fan Chen in UIC Analysis core	12-24	Not started		
Subtask 4: Analysis of Cohort 2 by Dr. Yi-Fan Chen	12-36	Not started		

Summary of Aim 1 Accomplishments:

Y1: Significant progress has been made on this aim with optimization of the method for testosterone metabolites in prostate tissues being completed (**Figure 1**). Optimization of detection of estrogen metabolites in prostate tissue is ongoing. Patient samples of tissue, sera and buffy coat have been collated for 48 patients and prepared for shipment to UPENN and COH.

		mg tissue	T pg/mg tissue	DHEA pg/mg tissue	DHT pg/mg tissue	Epi-AD fg/mg tissue	AD fg/mg tissue
S-13T-01	TURP tissue	45.6	1.19	15.2	0.03	12.18	5.06
S-13T-02	TURP tissue	50.9	1.09	11.5	0.02	3.00	11.90
S-14T-01	TURP tissue	52.3	0.15	6.7	1.29	477.16	224.82
S-14T-02	TURP tissue	54.1	0.13	7.1	1.08	423.37	241.53
S-15T-01	TURP tissue	44.3	0.14	5.9	0.55	351.80	90.33
S-15T-02	TURP tissue	48.5	0.11	11.1	0.91	382.40	165.61
S-16-01	RP tissue	58.1	0.01	0.7	0.08	403.10	86.01
S-16-02	RP tissue	55.2	0.09	0.2	0.06	175.49	63.92
S-17-01	RP tissue	42.6	0.03	4.5	0.06	269.27	240.81
S-17-02	RP tissue	57.6	0.02	4.4	0.14	297.28	419.21

Figure 1. Optimization of testosterone metabolite detection in human prostate tissues by LC-MS/MS (Penning Lab)

Y2: All samples have been collected for this Aim. This includes presurgical serum, DNDA and frozen tissue from 200 PCa patients. In the end, the cohort is comprised of 125 UIC patients and 75 from PCBN. Samples have been labeled and the first batch of 63 was sent to Penning in September of 2019.

There have been three unforeseen delays with this aim:

- MS/MS at U PENN as initially delayed due to personnel shortage
- SNP analysis at COP was delayed due to a lab shutdown and move
- Both are now delayed due to the covid-19 pandemic. Both institution are operating with single day shifts per week for each employee.

However, since all specimens are collected and methods optimized, we anticipate rapid progress as soon as the labs are functioning at capacity.

SPECIFIC AIM 2: To delineate the role of megalin-mediated endocytic transport in determining intracellular levels of androgens, estrogens and vitamin D in human primary prostatic cells from African-American men	Timeline (months)	UIC (Nonn) (Prins) (Chen) (Baumann)	UPenn (Penning)
Major Task 1: To determine if internalization of 25D, T and E2 in prostate epithelium is dependent on megalin	1-18		
Subtask 1: Obtain deuterated hormones (vitamin D, estrogens and androgens) and make sufficient volume of shRNA-LRP2 lentivirus	1-3	Completed	
Subtask 2: Conduct organoid experiments	3-18	In progress	In progress
Subtask 3: Ongoing analysis of findings as we replicate in N=10 AA PrE cells N=10 EA PrE cells	3-18	Not started	
Major Task 2: To examine the effects of vitamin D deficiency on megalin-mediated hormone endocytosis in prostate epithelium			
Subtask 1: Conduct organoid experiments	3-18	In progress	In progress
Subtask 2: Ongoing analysis of findings as we replicate in N=10 AA PrE cells and N=10 EA PrE cells	3-18	Not started	
Major Task 3: To determine if internalization of 25D, T and E2 in prostate epithelium is dependent on megalin in vivo	18-36		
Subtask 1: Induce megalin knockout in ER-Prob-cre, lrp2 ^{fl/fl} mice	18-24	In progress	
Subtask 2: Prostate harvest and send to Penning Lab for LC-MS/MS measurements of hormones	24-30	In progress	In progress
Subtask 2: Histological examination with Pathologist	24-36	In progress	

Summary of Aim 2 Accomplishments:

Y1: Optimization of the method to detect estrogens and androgens in the in vitro cells have been optimized (**Figure 2**) (Major Task 1). ShLrp2-lentivirus has been obtained for the organoids experiments (Major Task 1).

The 2 colonies of ER-Prob-cre and Lrp2-floxed mice are breeding well. Initial crosses began in June (Major Task 3).

			EpiT	Testosterone	DHEA	Androsterone	DHT	Epi-androsterone	5-androstenediol	3 α -androstenediol	3 β -androstenediol
S-01	cell media	EtOH	NF	1173	NF	1578	NF	NF	NF	1828	1671
S-02	cell media	10 nM T	NF	142907	NF	658242	NF	21569	NF	6187	NF
S-03	cell media	50 nM T	NF	651697	2300	3177334	NF	116135	NF	21424	381
S-04	cell media	10 nM E2	NF	14028	NF	76010	NF	2165	NF	135	118
S-05	cell media	50 nM E2	NF	2624	NF	18937	NF	NF	NF	424	180
S-06	cell pellet	EtOH	NF	2672	NF	327	NF	NF	NF	222	NF
S-07	cell pellet	EtOH	NF	82	NF	83	11	NF	NF	11	NF
S-08	cell pellet	EtOH	NF	266333	NF	117	54	NF	NF	264	NF
S-09	cell pellet	10 nM T	NF	140355	NF	31368	NF	NF	580	1377	829
S-10	cell pellet	50 nM T	NF	224413	NF	143210	NF	NF	3200	6959	269
S-11	cell pellet	10 nM E2	NF	1761	NF	82	34	NF	NF	NF	NF
S-12	cell pellet	50 nM E2	NF	31050	NF	128	1284	NF	NF	NF	NF

NF=not found

Figure 2. Optimization of testosterone and estrogen metabolite detection in patient derived cell cultures by LC-MS/MS (Penning Lab)

Y2:

Optimized vitamin D import assays: In partnership with Heartland Assays, we optimized the dose and timing of 25D to quantify import into the organoids (data not shown). In Y3 we will complete the assays, along with the T and E2 optimized in Y1, in the organoids.

Knockout of Lrp2 in prostate organoids: We encountered difficulties with the shLrp2 lentivirus. Although we have good transduction, the knockdown of Lrp2 is minimal. We suspect that there are splice variants in the prostate that do not respond to the nucleic acid sequences we have tried. Lrp2 is 80 exons, thus multiple splice variants are highly likely, but have yet to be reported. We are mapping the cDNA and designing new sequences. As all endpoints have been optimized, the experiments with the patient-derived organoids will progress quickly once we have the correct shLrp2 sequences.

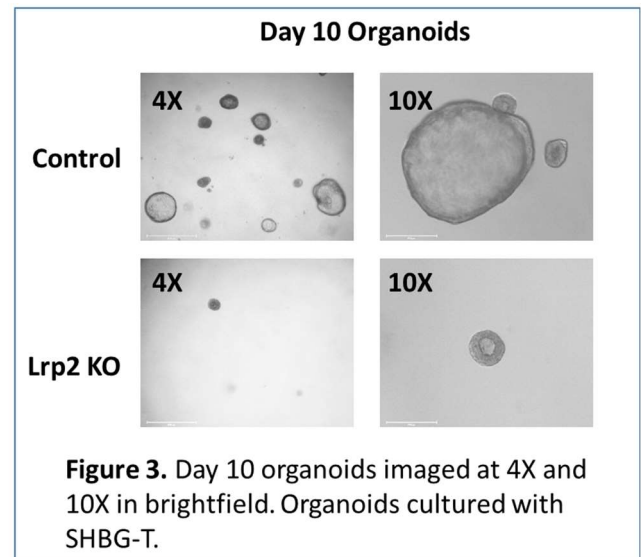


Figure 3. Day 10 organoids imaged at 4X and 10X in brightfield. Organoids cultured with SHBG-T.

In the meantime, we have utilized our Lrp2KO mice for the organoid experiments. Organoids without Lrp2 have profound reduction in size, number and shape (**Figure 3**). These were cultured in standard organoid medium with SHBG-T used instead of DHT, to require Megalin for import. Ongoing experiments will complete these experiments in the presence of vitamin D deficiency.

Knockout of Lrp2 in mouse prostate: The crossed bitrans $Lrp2^{fl/fl}/Pr-Mer-Cre-Mer$ mouse line has been established and is breeding well. We discovered unexpected side effects of tamoxifen treatment (to induce cre) on the male reproductive organs and had to modify our protocols and endpoints accordingly. These findings have been minimally reported in the literature, but we will include in our publication. Tamoxifen induced a complete seminal vesicle atrophy that takes 6 weeks to recover.

We switched to treating pups with tamoxifen days 10-15. The prostates were collected at 8 weeks and showed atrophy of the prostate only in the $lrp2^{fl/fl}/cre$ mice compared to $lrp2^{fl/fl}$ alone (**Figure 4**). All mice were treated with tamoxifen to control for hormonal effects of induction.

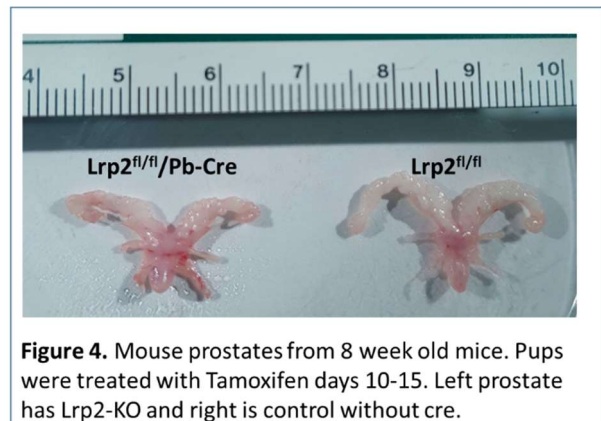
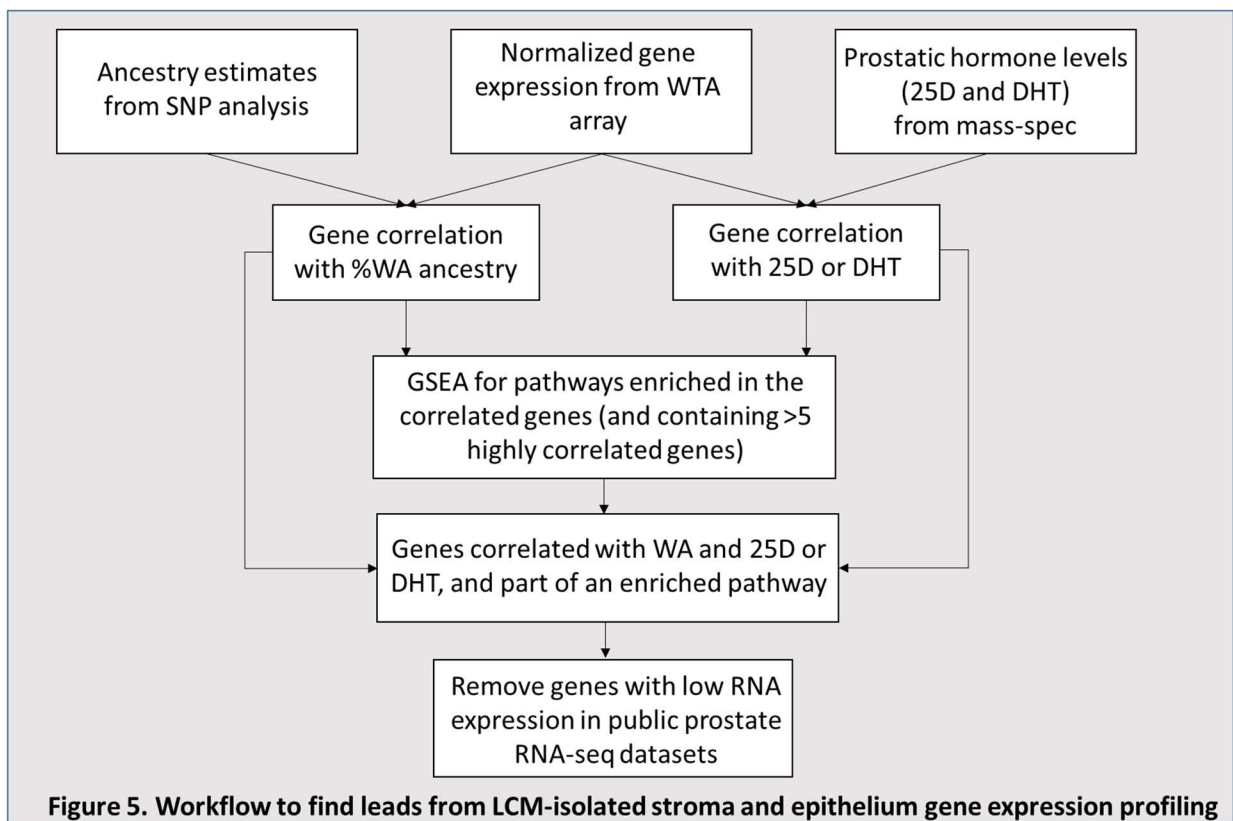
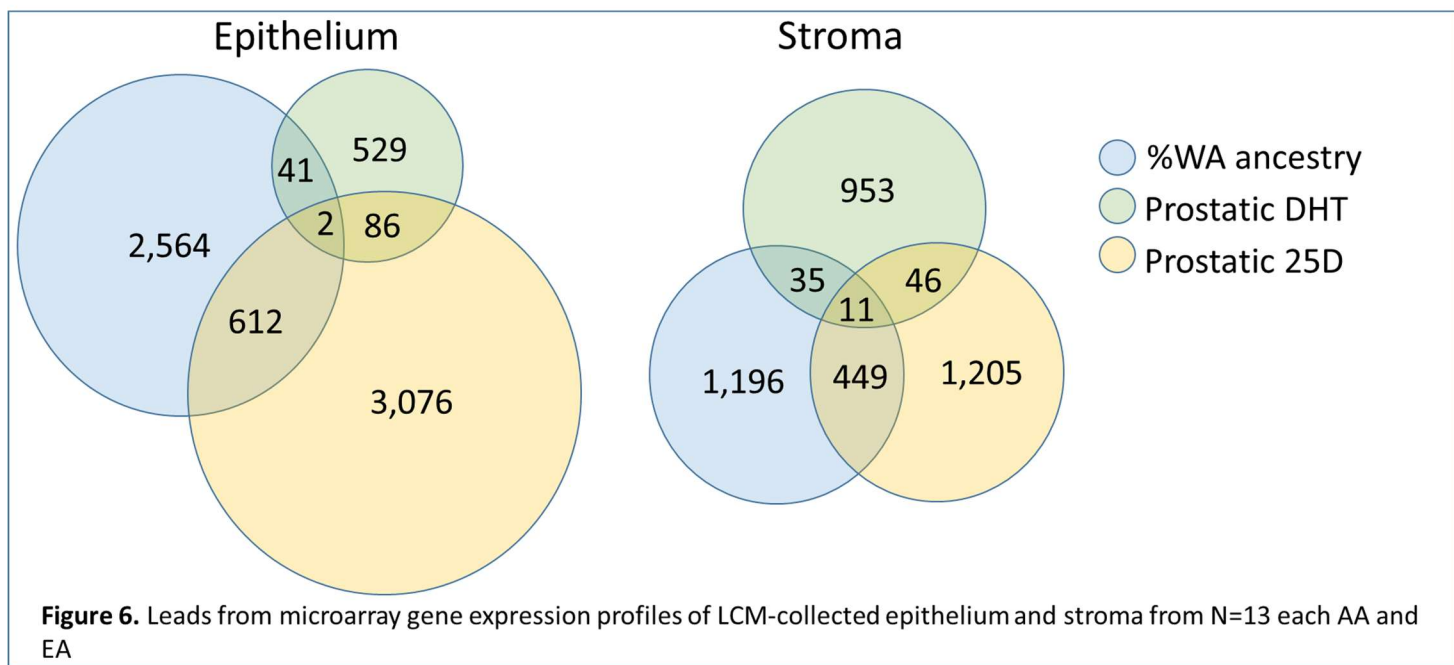


Figure 4. Mouse prostates from 8 week old mice. Pups were treated with Tamoxifen days 10-15. Left prostate has Lrp2-KO and right is control without cre.

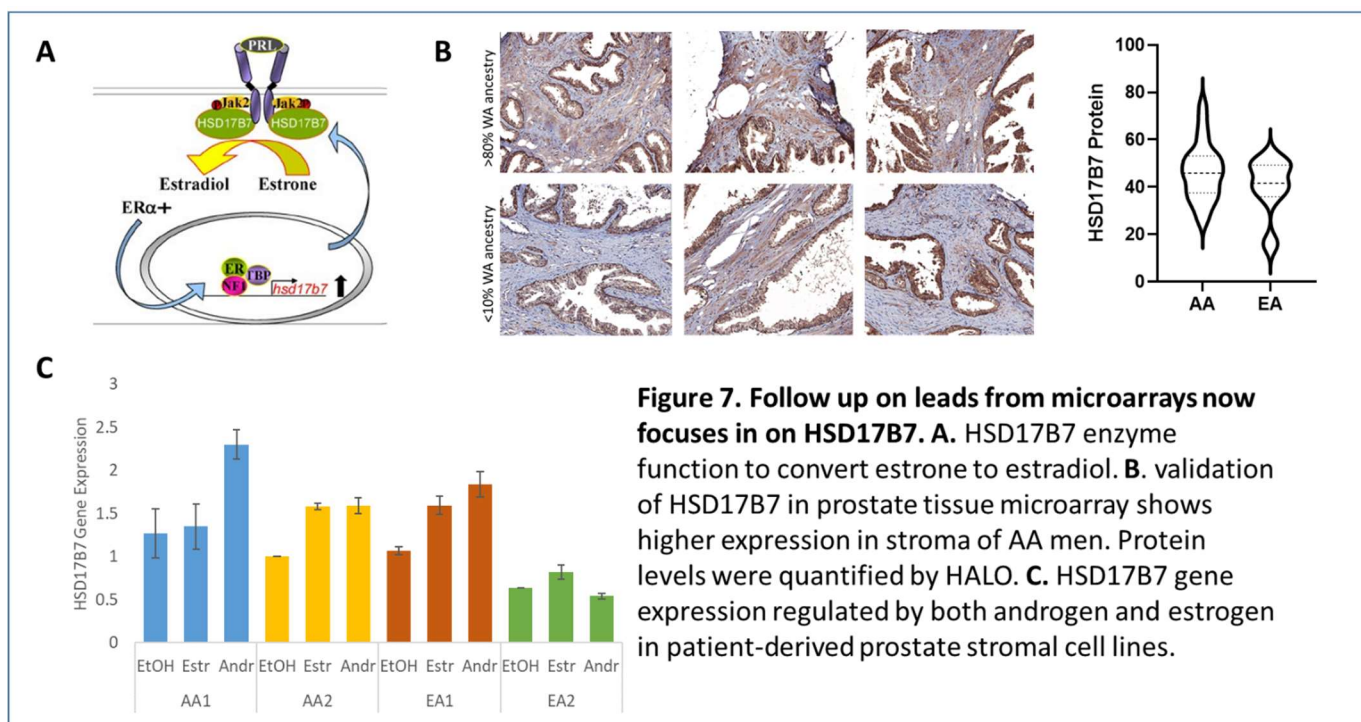
SPECIFIC AIM 3: To identify cancer risk pathways that associate with intra-prostatic hormone concentrations in prostate epithelium and stroma from African American and European American men	Timeline (months)	UIC (Nonn) (Prins) (Chen) (Baumann)
Major Task 1: Complete Affymetrix Arrays of LCM-isolated prostate stroma and identify cancer-related pathways that associate with hormone and megalin status	1-18	
Subtask 1: Select patients and run the arrays (Baumann)	1-6	Complete
Subtask 2: Data analysis and comparison to epithelial array data	6-12	Complete
Subtask 3: Data analysis and comparison to Cohort 1 full patient data included T and E measurements (Specific Aim 1)	6-18	In progress
Major Task 2: 2B: To examine megalin-mediated effects on organoids	18-36	
Subtask 1: Design PCR primers and pathway phenotypes	18-24	In progress
Subtask 2: Conduct organoid experiments using shRNA to knockdown LRP2	24-36	In progress

Summary of Aim 3 Accomplishments: Y1: The microarray on the stroma was completed as well as analysis of the data (Major Task 1). The gene expression from the arrays was analyzed with the prostatic levels of vitamin D (25D) and DHT as well as the % of west African ancestry (**Figure 5**). Several leads were generated from the correlations between hormone levels and gene expression (**Figure 6**). Several of the genes were validated by PCR and expression in patient tissues, including HSD17B7 in stroma, required for biosynthesis of sex steroids (**Figure 7A-B**).





Y2: After further validation we have focused in on stromal HSD17B7 as a top differentially expressed gene that is correlated with both vitamin D and androgens in the prostate. This enzyme mediates conversion of estrone to estradiol (**Figure 7a**). We quantified the TMA that was stained in Y1 using HALO software and found HSD17B7 to be higher in the stroma of AA men compared to those of European descent (EA) (**Figure 7b**). Additionally, validation in patient-derived prostate stromal cells from AA and EA men showed regulation of HSD17B7 gene expression by both estrogen and androgen (**Figure 7c**).



- **What opportunities for training and professional development has the project provided?**

The project provides training for postdoctoral fellow, Dr. Bethany Baumann, who is in Dr. Nonn's lab.

Training Activities:

One-on-one work with Dr. Nonn to go over data and prepare reports
Mentorship of Julian Pacheco, formerly an undergraduate in the lab and now a medical student, on the project

Methods learned:

Analysis of gene expression profiling data
Pathway analysis (GSEA and Ingenuity)
RT-qPCR
R statistical programming
Patient-derived prostate organoid culture

Professional Development:

Informatics training by UIC Bioinformatics core on analysis of gene expression
Attendance to the Society for Basic Urology Research Conference, Fall 2018 and 2019
Attendance to American Urological Association Conference, Spring 2019
Attendance to local Chicago AUA Conference, Fall 2019
University of Illinois at Chicago Bioinformatics Certificate
Responsible Conduct of Research seminars on Mentoring, Conflict of Interest and Detrimental Research Practices.
University of Illinois at Chicago Your Future in Science seminars on Negotiations, Data Management, and Social Media in Science
University of Illinois at Chicago Postdoctoral Association Workshops on Leadership and Developing Transferrable Skills

• **How were the results disseminated to communities of interest?**

Nothing to Report.

Multiple Poster sessions at local and national conferences:

- College of Medicine Research Day 2
- Society for Basic Urology Research Conference
- UIC Cancer Center Research Day
- Vitamin D Workshop (promoted oral presentation)

• **What do you plan to do during the next reporting period to accomplish the goals?**

We are on track with the SOW. We plan to ship another 100 samples to Penning and Heartland for hormone measurements. The mouse model will be complete and hormone levels measured. The organoids studies commenced.

4. IMPACT:

• **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to report

• **What was the impact on other disciplines?**

Nothing to report at this time.

- **What was the impact on technology transfer?**

Nothing to report

- **What was the impact on society beyond science and technology?**

Nothing to report

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Delays in MS/MS and SNP analysis are detailed in the aims above. These are ongoing due to reduced personnel at UPenn and COP. Since all samples are collected, we hope to complete the hormone measurements quickly once they are up and running.

- **Changes that had a significant impact on expenditures**

The pandemic has reduced our supply expenditures for 2020

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

- **Significant changes in use or care of human subjects**

Nothing to report.

- **Significant changes in use or care of vertebrate animals.**

Nothing to report.

- **Significant changes in use of biohazards and/or select agents**

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
Website(s) or other Internet site(s)
Technologies or techniques
Inventions, patent applications, and/or licenses
Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?

Name:	Larisa Nonn
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.8
Contribution to Project:	Dr. Nonn oversaw all aspects of this proposal and coordinated samples transfer between the UIC and UPENN
Funding Support:	NA

Name:	Gail Prins
Project Role:	Co-I (Qualified Collaborator)
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.2
Contribution to Project:	Dr. Prins provided expertise on hormone metabolism and signaling. Participating in all project meetings and looking over project data
Funding Support:	NA

Name:	Michael Schlicht,
Project Role:	Research Specialist
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	9
Contribution to Project:	Mr. Schlicht oversees the mouse colonies and creates ll primary patient-derived cell cultures
Funding Support:	NA

Name:	Bethany Baumann
Project Role:	Nested Young Investigator (postdoc)
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.6
Contribution to Project:	Dr. Baumann performed all gene expression analyses and validation experiments.
Funding Support:	NA

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

- **What other organizations were involved as partners?**

Organization Name: University of Pennsylvania

Location of Organization: USA

Partner's contribution to the project:

Collaboration: this project is collaborative with Dr. Penning. His role and effort, as well as that of his postdoctoral fellow, were detailed in the original funded proposal.

Name:	Trevor Penning
Project Role:	Co-I (UPENN)
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6 calendar months
Contribution to Project:	Dr. Penning is oversaw the LC-MS/MS analysis of the prostate tissue and cells.
Funding Support:	NA

Name:	Rajesh Kishore Sanku
Project Role:	Postdoctoral fellow UPENN
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3 calendar months
Contribution to Project:	Dr. XX conducted the isolation and quantitation of the hormones by LC-MS/MS analysis in the prostate tissue and cells.
Funding Support:	NA

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Gail Prins is the qualified collaborator on this project. Both Drs Nonn and Prins are at UIC and only one report was submitted.