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TITLE: Detection and Characterization of Treatment Emergent Neuroendocrine Prostate Cancer Using Hyperpolarized ¹³C Magnetic Resonance

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Fort Detrick, Maryland 21702-5012**

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14. ABSTRACT The goal of this proposal is to discriminate biologically divergent treatment-emergent neuroendocrine prostate cancer (NEPC) from advanced adenocarcinoma based on the metabolic profile of NEPC tumors and to use real-time changes in metabolism to monitor therapeutic efficacy by using novel hyperpolarized (HP) ¹³ C metabolic imaging techniques. In the 1 st year of funding, our major activities focused on collating preliminary studies for Aims 1 and 2 to establish feasibility. To this end for Aim 1, the probes (glutamine and acetate) were optimized for hyperpolarization technique and tested in cells and baseline TRAMP studies. Correlative measures of NEPC presence were developed using ELISA and serum enzyme activities in addition to IHC quantification. Furthermore preliminary metabolic profiling of TRAMP tumors showed altered substrate utilization with the onset of CRPC further strengthening our hypothesis of metabolic adaptations with treatment resistance. For Aim2, we did exhaustive feasibility of labeling studies in biopsy samples, and for the first time showed viability and detected ex vivo measures of glycolytic activity.					
15. SUBJECT TERMS Hyperpolarized ¹³ C MRI, pyruvate, treatment emergent neuroendocrine prostate cancer, metabolism					
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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The **clinical motivation** driving this project is the critical, unmet need to discriminate the biologically divergent, treatment-emergent neuroendocrine prostate cancer (NEPC) from advanced adenocarcinoma. To achieve the study goals, novel Hyperpolarized (HP) ^{13}C magnetic resonance imaging (MRI) technique to detect real-time changes in metabolism with disease progression from castrate-sensitive to castrate-resistance and eventually to treatment-emergent neuroendocrine prostate cancer as well as to monitor therapeutic efficacy was performed. A gap in knowledge hindering the management of t-NEPC, is the lack of information concerning the genomic drivers and subsequent metabolic processes regulating t-NEPC. To this end, high-resolution NMR metabolic profiling via both isotopomer analysis as well as steady-state concentrations of metabolites was performed in conjunction with HP studies in clinically pertinent models.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Magnetic Resonance Imaging (MRI), Hyperpolarized ^{13}C MRI, dissolution dynamic nuclear polarization, glycolysis, prostate cancer, transgenic adenocarcinoma of the murine prostate (TRAMP), spectroscopy, metabolism, metabolites, biopsy, stable-isotope resolved metabolomics (SIRM)

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Establish the molecular and metabolic signature of t-NEPC tumors and develop new HP ^{13}C labeled probes to identify neuroendocrine differentiation and its response to therapy.

Specific Aim 2: Define the molecular and metabolic signature of metastatic NEPC tumors from patient biopsy samples.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Specific Aim 1:

- 1) Characterize new HP ^{13}C labeled probes: Although we were able to characterize the T_1 and polarization of hyperpolarized $[5-^{13}\text{C}]$ glutamine and $[1-^{13}\text{C}]$ acetate (detailed in first years progress report), we were not able to observe any metabolism in the TRAMP mice. However, we were able to measure the glycolytic capacity of the TRAMP tumor progression upon treatment (both castration as well as ARN-509) using hyperpolarized $[1-^{13}\text{C}]$ pyruvate.

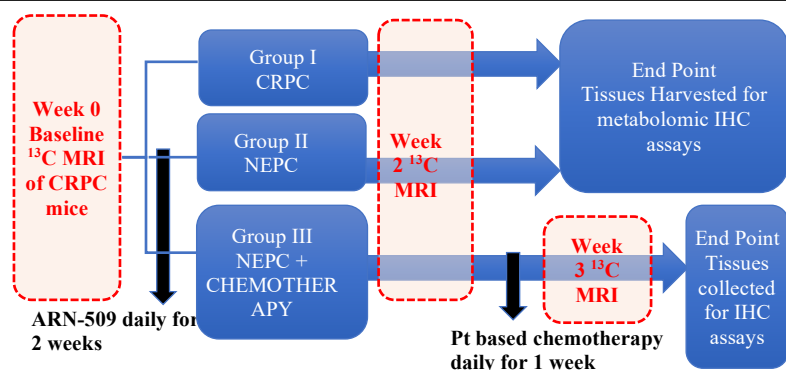


Figure. 1: Timeline of animal model development and imaging

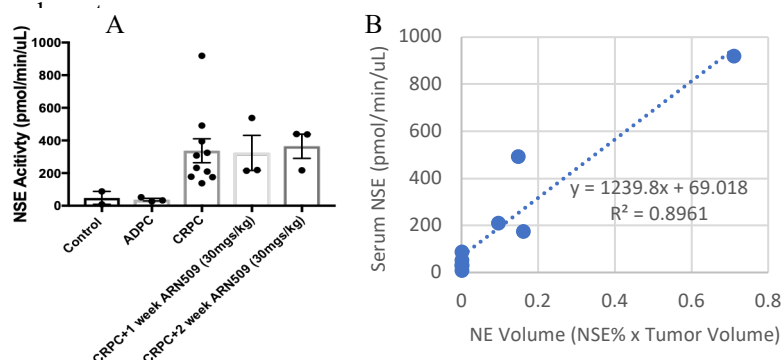


Figure. 2: A) Serum NSE activity with development of CRPC and subsequent ARN-509 treatment. B) Correlation between serum NSE activity (y-axis) and mean NEPC staining (x-axis, synaptophysin).

found that in untreated (control mouse prostate) and in androgen dependent TRAMP tumors, there was minimal NSE activity which increased dramatically with development of CRPC and subsequent treatment with ARN509 (**figure 2A**).

- b. We subsequently established a quantitative image analysis toolkit (using Matlab detailed in Appendix 1) to quantify the amount of neuroendocrine cells represented as % positive stained cells of appropriately thresholded synaptophysin stained TRAMP tumor sections (4 sections from throughout the tumor). 4 Using this approach we were able to correlate the serum NSE activity to calculated neuroendocrine (NE) cell volume based on the quantitative synaptophysin staining of TRAMP tumors at the end of the imaging exam (**figure 2B**). We are now able to use the serial serum NSE activity measurement during TRAMP tumor progression as a non-invasive means to assess neuroendocrine burden.

- 3) HP imaging of TRAMP tumor progression and metabolomics: The genetically modified TRAMP mouse model of prostate cancer, provides a unique model mirroring the tumor progression in response to primary androgen deprivation therapy. The trial schema outlined above (**figure 1**) starts with the establishment of castration-resistant prostate cancer (CRPC) in TRAMP tumors, defined in our study as tumor growth of more than 20% ~ 1 week post orchiectomy and the mice with less than 20% increase in tumor volume was designated as androgen dependent prostate cancer (ADPC). With subsequent addition of second line anti-androgens and chemotherapy spanning 4 weeks after orchiectomy. Owing to the logistical difficulties and loss of mice due to morbidity of repeated anesthesia and cannulation, the study was broken down into three phases to achieve tangible outcomes using hyperpolarized [$1\text{-}^{13}\text{C}$]pyruvate to assess the change in glycolysis as a marker of therapeutic efficacy or tumor progression

- a. **Development of CRPC:** In the first phase we established that there was a significant increase in glycolysis as measured by a increase in the HP lactate to pyruvate ratio (Lac/Pyr) in *in vivo* ^{13}C tumor spectra after administration of hyperpolarized [$1\text{-}^{13}\text{C}$] pyruvate in TRAMP tumors that became castration-resistant (CRPC) relative to androgen sensitive TRAMP tumors (ADPC) (Figure 3). In conjunction with this, we also performed extensive metabolomic assessment in these TRAMP tumors as well as in androgen sensitive and castrate resistant human cell lines which is to be published (Appendix 2) for a special edition of the journal *Metabolites*, entitled "Applications of Magnetic Resonance (MR)-Based Metabolic Imaging in Medicine". This metabolomic study

2) Model Development:

- a. Aim 1 is centered around the genetically engineered mouse model of TRAMP (Transgenic Adenocarcinoma of the Mouse Prostate), which upon treatment develops NEPC similar to the clinical setting according to the schema presented in **figure 1**. CRPC mice are defined as those tumors that have >20% increase in tumor volume 5 days ($\pm$ 2 days) post orchiectomy. While clinical serum neuroendocrine markers are typically used to determine the presence of neuroendocrine prostate cancer for appropriate treatment selection, we needed a quantitative serum protocol to determine both the development and amount of NEPC in TRAMP tumors. This was critical to our studies since determining the NEPC tumor burden through pathologic staining of the murine tumor post imaging would have resulted in the need to study a very large number of mice to get sufficient data on the relationship between NEPC and metabolism. To accomplish this we used neuron specific enolase (NSE) enzyme activity of the mouse serum as a marker of NEPC development and tumor burden and

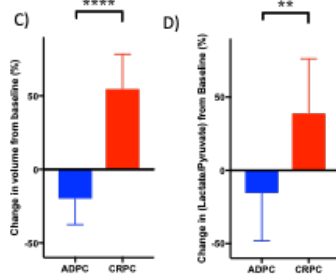
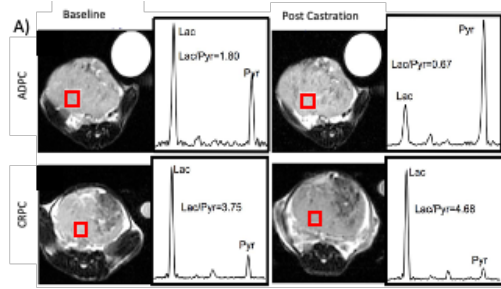


Figure 3. A, Representative T2-weighted proton images of ADPC (top row) and CRPC (bottom row) TRAMP mouse at baseline (left column) and 5 days post-castration (right column). The spectrum from a voxel (red square) is shown on the right of each image of the hyperpolarized lactate (Lac) and pyruvate (Pyr) and the calculated ratio (Lac/Pyr). B, Change in volume and C, hyperpolarized Lac/Pyr of TRAMP tumors from baseline to post-orchietomy in ADPC (n=11) and CRPC (n=7) mice.

demonstrated a significant increase in glycolysis as well as in the TCA cycle in CRPC relative to androgen sensitive prostate cancer.

- b. Treatment of CRPC with ARN-509:** In the second phase, ARN-509 (apalutamide) the second-line anti-androgen was given at 10 mg/kg orally daily to the CRPC TRAMP mice and imaged with

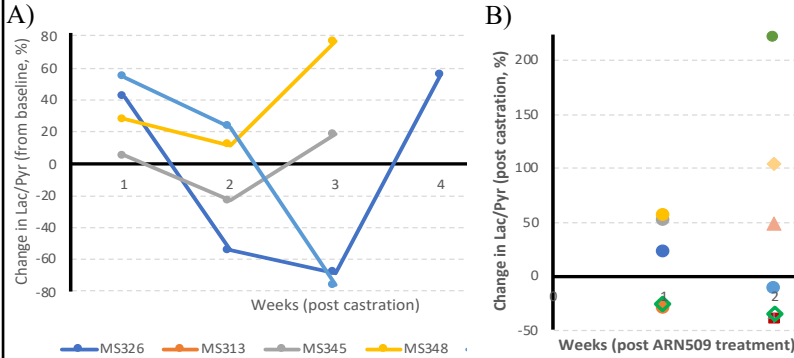


Figure 4. A, Change in HP Lac/Pyr over time from baseline (%) post castration and 10 mg/kg ARN509 treatment in CRPC TRAMP mice. B, Change in Lac/Pyr post castration (%) at week 1 (n=5) and week 2 (n=6) post 30 mg/kg ARN509 treatment.

HP [^{13}C]pyruvate serially for 3 weeks after orchiectomy and subsequent development of CRPC. Figure 4A shows that in the four TRAMP mice that were imaged, glycolysis decreased (decreasing HP Lac/Pyr) for the first 2 weeks of ARN-509 treatment indicating therapeutic efficacy. However, the subsequent increase in HP Lac/Pyr at 3 weeks post castration indicated the development of resistance to ARN-509 and this imaging finding was verified by increased

LDH activity and reduced tumor growth rate. However, the quantitative assessment of synaptophysin staining of tumor tissue at the end of the imaging study demonstrated that NEPC tumor burden had not significantly increased at this dose of ARN-509 and time of treatment. In order to abbreviate the time to therapeutic resistance and to increase the amount of NEPC development, the subsequent cohort of TRAMP mice were given an increased dose of 30 mg/kg daily. Hyperpolarized [^{13}C]pyruvate imaging in this cohort of mice demonstrated increased hyperpolarized ^{13}C Lac/Pyr ratio in approximately 50% of the mice indicative of the development of NEPC with resistance to ARN509 treatment (Figure 4B). At the beginning of the 2020, we had started investigating the effect of 60 mg/kg ARN-509 in order to further to increase the amount of therapeutically driven NEPC development but had to abandon after 3 mice due to shutdown of research activities during the COVID pandemic.

- c. Treatment of CRPC with ARN-509 + Carboplatin:** Figure 5A shows a representative hyperpolarized ^{13}C MR tumor spectrum taken from a TRAMP mouse who had received 1 cycle of 30mg/kg ARN509 followed by carboplatin treatment. There was a consistent clear reduction in hyperpolarized [^{13}C]lactate relative to [^{13}C]pyruvate after carboplatin treatment relative to ARN509 treatment alone. Treatment with carboplatin also resulted in a dramatic reduction in tumor growth rate relative to ARN-509 treatment alone (figure 5B). The dynamic hyperpolarized ^{13}C data was used to calculate the rate of conversion of [^{13}C]pyruvate to [^{13}C]lactate, k_{PL} , which was reduced after carboplatin treatment (figure 5C). The dynamic hyperpolarized ^{13}C data was used to calculate the rate of conversion of [^{13}C]pyruvate to [^{13}C]lactate, k_{PL} , which was reduced after carboplatin treatment (figure 5C). Metabolomic studies using stable isotope resolved metabolomics, the rate of glycolysis and TCA cycle activity, measured by the fractional enrichment of the pools of lactate and glutamate and aspartate were significantly reduced after carboplatin treatment relative to ARN509 treatment alone and were similar to these metabolic fluxes in androgen sensitive prostate cancer (figure 5D).

- 4. Metabolomics of CRPC and NEPC PDX studies:** While we were able to drive TRAMP tumors to develop NEPC similar to clinical progression (see Appendix - RNA seq analysis differentiating CRPC and NEPC

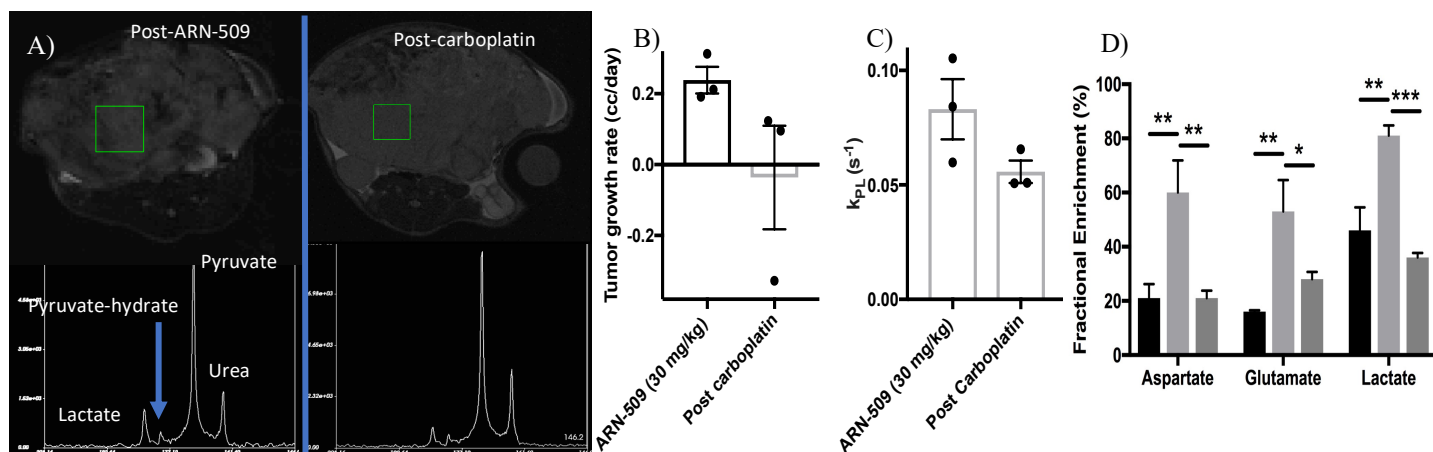


Figure 5. A) The top row is the T2 weighted proton images and the bottom row shows the hyperpolarized ^{13}C spectrum from a single tumor voxel (green box). B) Tumor growth rate changes and C) k_{PL} in carboplatin treated mice relative to those that received ARN-509 alone. D) The fractional enrichment, shows a reduction in glucose metabolism and glycolysis in the ARN509 treated TRAMP (dark gray bars) mice in comparison to the castrate resistant (light gray bars) and more similar to the castrate sensitive TRAMP (black bars).

TRAMP tumors), the magnitude of NEPC transdifferentiation in the TRAMP model was not as high as that observed in patients. To address this problem, we performed stable isotope resolved metabolomic studies of patient derived CRPC and NEPC xenografts. We found that CRPC (LTL-313HR, LuCaP 70CR, LuCaP 77CR) and NEPC (LTL-352, LTL-610, LuCaP 93, LuCaP 145.1) PDX tumors had distinct metabolic profiles as discerned by principal component analysis (PCA)(figure 7) based on both glucose and glutamine metabolic pathways. Specifically, it shows that NEPC tumors have revved up glycolysis and utilization of both glucose and glutamine contributing to increased TCA cycle activity. Details of this study can be found in appendix 4.

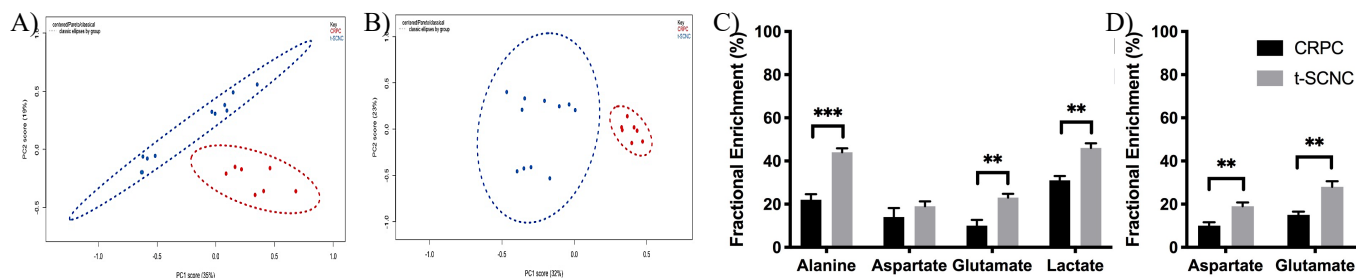


Figure 6. PCA loading plots of CRPC (blue) and NEPC (red) of A) ^{13}C -glucose labeled and B) ^{13}C -glutamine labeled tumor extracts. Fractional enrichments of the same tumors of C) ^{13}C -glucose and D) ^{13}C -glutamine labeling.

Specific Aim 2: Although we were not able to procure enough material from the metastatic biopsies from the WCDT cohort, (which is of limited availability and shared over a large number of researchers) to enable the measurement of metabolic fluxes in these samples, we have for the first time shown that it is possible to use stable isotope resolved metabolomics to study differential pathway activation in biopsy samples using primary prostate cancer tissue. This has resulted in the manuscript titled “NMR quantification of lactate production and efflux and glutamate fractional enrichment in living human prostate biopsies cultured with $[1,6-^{13}\text{C}_2]\text{glucose}$.” *Magn Reson Med* **2019**, 82, 566–576 (appendix 5).

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional

development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The doctoral students (Sun and Brown) and postdoctoral scholar (Dr. Agarwal) benefitted from training under Dr. Sriram’s expertise in various aspects of this study that included stable isotope resolved high resolution NMR based metabolomics, hyperpolarized carbon-13 imaging. Furthermore, Dr. Sun and Dr. Agarwal, learnt about the intricacies of TRAMP tumor anatomy from Dr. Bob Bok.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The results are disseminated via publications, (two are already published and two more are in the preparation). Additionally for opportune sharing of important findings, these studies were showcased in various professional organization’s annual meeting, such as Experimental NMR conference (ENC), International Society of Magnetic Resonance in Medicine (ISMRM). Some of this work was also presented at the annual prostate cancer meeting at our institution in which patient representatives are active participants.

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

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to report

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The biggest impact from the preliminary studies, has been

- i. A rational approach to developing hyperpolarized biomarkers. For example, using the metabolomics data from the TRAMP labeling studies, it seems that using HP pyruvate may show a larger % change in glutamate signal than when using HP glutamine and maybe a better alternative, considering the poor performance in terms of polarization and T1 characteristics of the latter probe.
- ii. We have shown for the first time, that it is feasible to keep biopsy tissue viable and measure the glycolytic process *in situ*. We have developed methodology for the same that can be used to optimize such measures for other tissues. To facilitate this, detailed procedures will be published in a manuscript shortly.
- iii. We have developed preclinical tools such as a) quantitative imaging of immunohistochemical

stains of neuroendocrine cells, b) serum assays for neuron specific enolase activity, and c) correlation of the two metrics as a means of non-invasively assessing the presence of neuroendocrine prostate cancer with disease progression.

- iv. We have shown that while TRAMP may not be an ideal model to use for the development of imaging biomarkers for disease transdifferentiation, we have clearly shown the metabolic adaptations of CRPC and NEPC and how it can be used to develop imaging biomarkers.
- v. We have shown that HP metabolic imaging of pyruvate's conversion to lactate can be used as a robust marker of therapeutic efficacy for both orchiectomy (first line anti-androgen therapy), ARN-509 (second-line anti-androgen therapy) as well as carboplatin (chemotherapy).

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The methodical approach of using stable isotope metabolomics to discern the altered pathways in the disease of interest is applicable to most pathologies and can help increase the success of development of metabolic imaging biomarkers.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions;*
or
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Towards the last year of the project, as soon as we realized the lack of robust development of neuroendocrine tumors in the TRAMP mice complicated with very high mortality and morbidity associated with treatment, we started using patient derived xenografts to assess the metabolic adaptations of NEPC tumors.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

With the onset of the pandemic in March 2020 and the associated curtailment of research activities, we were unable to do any imaging from March 15th until September 15th as we were precluded from obtaining new animals into the facility. This was further complicated by the quenching of our 3T magnet and subsequent deenergizing of the magnet for 4 months during the same period (in addition to 3 months in early 2019).

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Brown, J. B.; Sriram, R.; VanCrickinge, M.; Santos, R. D.; Sun, J.; Santos, J. D.; Tabatabai, Z. L.; Shinohara, K.; Nguyen, H.; Peehl, D. M.; Kurhanewicz, J. NMR quantification of lactate production and efflux and glutamate fractional enrichment in living human prostate biopsies cultured with [1,6-13C2]glucose. *Magn Reson Med* **2019**, 82, 566–576.
Acknowledgement of federal support: yes
2. Jinny Sun, Robert A Bok, Justin Delos Santos, Deepti Upadhyay, Romelyn Delos Santos, Mark Van Crickinge, Daniel B. Vigneron, Rahul Aggarwal, Donna M. Peehl, John Kurhanewicz, and Renuka Sriram "Resistance to Androgen Deprivation Leads to Altered Metabolism in Human and Murine Prostate Cancer Cell and Tumor Models. *Metabolites*. 2021. Special issue: "Applications of Magnetic Resonance (MR)-Based Metabolic Imaging in Medicine". (awaiting publication).
Acknowledgement of federal support: yes

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Dissertation of students, now Dr. Jinny Sun and Dr. Jeremy Bancroft Brown
2. Chapter 6 entitled "**Using Hyperpolarized NMR to Understand Biochemistry from Cells to Humans**", in the book *Handbook of Modern Biophysics*, , Thomas Jue and Dirk Mayer (Eds): *Dynamic Hyperpolarized Nuclear Magnetic Resonance*
Acknowledgement of federal support: yes

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Invited talk - Podium presentation of "Development of Hyperpolarized 13C metabolic biomarkers of prostate cancer aggressiveness - from cells to patient derived tissues". The Fifth International Workshop on Metabolic Imaging, University of Pennsylvania, PA, USA 2018.

Sun J, **Sriram R**, Bok R, Delos Santos R, Delos Santos J, Van Crickinge M, Vigneron D, Kurhanewicz J. "Sensitive, High-Resolution 1D and 2D NMR for 13C Metabolomics Studies of Small-Abundance Mouse Tissue". Experimental NMR conference, 2018, Poster Presentation

*Brown JB, Subramaniam S, Van Crickinge M, Delos Santos R, Nolley R, Sun J, Delos Santos J, Tabatabai L, Shinohara K, Nguyen H, Peehl D, **Sriram R**, Kurhanewicz J. "NMR Quantification of Glucose Consumption and Lactate Efflux in Living Human Prostate Biopsies". Experimental NMR conference, 2018, Oral Presentation

*Sun J, Delos Santos J, Bok R, Delos Santos R, Van Crielinge M, Vigneron DB, **Sriram R**, Kurhanewicz J. "Androgen Independence Leads to Altered Metabolism in Prostate Cancer Cell and Murine Models". Oral Presentation at the annual meeting of International Society of Magnetic Resonance in Medicine, 2019, Montreal, Canada.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

A MATLAB routine was developed for quantification of immunohistochemical stain.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Dr. Renuka Sriram : no change
Dr. John Kurhanewicz : no change
Dr. Rahul Aggarwal : no change
Dr. Robert Bok : no change
Dr. Shubhangi Agarwal: no change
Fayyaz Ahamed: no change
Romelyn DeLos Santos : no change
Seth Vigneron : no change

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner's contribution to the project (identify one or more)

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other.

"Nothing to Report."

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

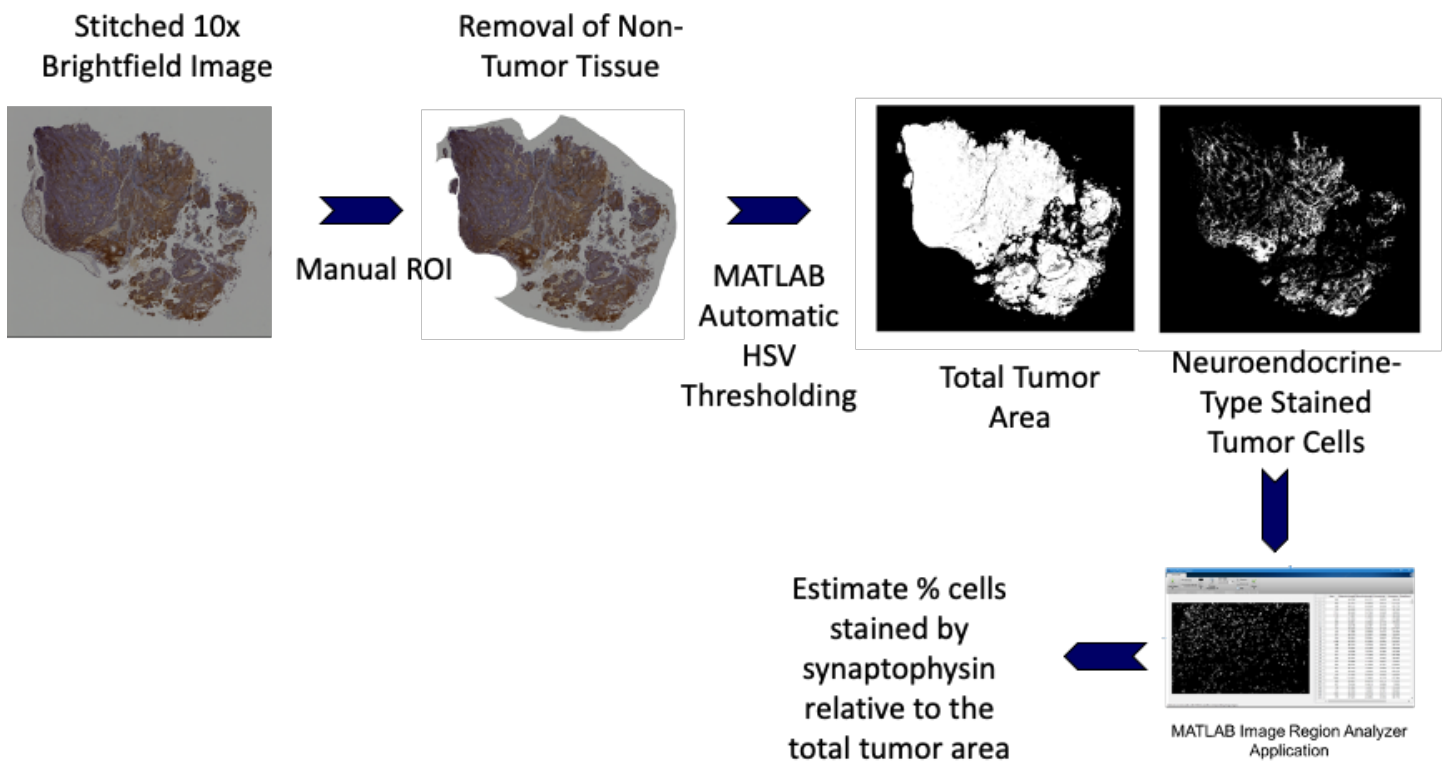
9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Appendix:1

Quantification of the % Synaptophysin staining

Tumor sections were stained for NEPC using NSE and synaptophysin stains. Images of the tumor sections were made by stitching 10x Brightfield images (Nikon 6D high throughput scope) together. Homebuilt MATLAB scripts were used to threshold the images based on pixel values to quantify the NEPC staining.

For the Synaptophysin stained images (figure below shows the processing pipeline), hue values on the interval of $[0, 0.169]$ to capture the brown and the interval of $[0.189, 1.000]$ for the saturation values were used to threshold in the HSV color space after manually segmenting the prostate gland on the slide. Due to differing illumination from image to image, the allowed value values ranged from the 0th percentile to the 16.86th percentile. Next, image regions were analyzed from the resulting binary image, and the total pixel count of the detected regions was stored. The same protocol was adapted on the region of the HSV color space needed to select the background in every image: Hue = $[0.113, 0.738]$, Saturation = $[0, 0.161]$, Value from 71.69th percentile to 100th percentile. After running the functions to detect the pixel count of Synaptophysin expression pixels and background pixels, the detected expression pixel count was divided by the total pixel count minus the background pixels to give the percentage Synaptophysin expression. The values were then averaged for each specimen.



Article

Resistance to Androgen Deprivation Leads to Altered Metabolism in Human and Murine Prostate Cancer Cell and Tumor Models

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Abstract: Currently no clinical methods reliably predict the development of castration-resistant prostate cancer (CRPC) that occurs almost universally in men undergoing androgen deprivation therapy (ADT). Hyperpolarized (HP) ¹³C magnetic resonance imaging (MRI) could potentially detect the incipient emergence of CRPC based on early metabolic changes. To characterize metabolic shifts occurring upon the transition from androgen-dependent to castration-resistant prostate cancer (PCa), the flux of [U-¹³C]glucose and [U-¹³C]glutamine through glycolysis, the tricarboxylic acid (TCA) cycle, glutaminolysis, and glutathione synthesis in human PCa cell lines and the TRAMP murine model of PCa was analyzed by nuclear magnetic resonance (NMR) spectroscopy. Comparison of steady-state metabolite concentrations and fractional enrichment in androgen-dependent LNCaP cells and TRAMP tumors versus castration-resistant PC-3 cells and treatment-driven CRPC TRAMP tumors demonstrated that CRPC was associated with upregulation of glycolysis, TCA metabolism of pyruvate and glutamine, glutaminolysis, and glutathione synthesis. These findings were supported by: ¹³C isotopomer modeling using TCACALC showing increased flux through pyruvate dehydrogenase (PDH) and anaplerosis; enzymatic assays showing increased lactate dehydrogenase (LDH) activity, PDH activity, and glutaminase (GLS) activity; and oxygen consumption measurements showing increased dependence on anaplerotic fuel sources for mitochondrial respiration in CRPC. HP [1-¹³C]pyruvate NMR spectroscopy distinguished androgen-dependent PCa from CRPC in cell and tumor models.

Keywords: Metabolism, prostate cancer, castration resistant, androgen dependent, TRAMP, magnetic resonance, hyperpolarized [1-¹³C] pyruvate, glycolysis.

1. Introduction

Androgen deprivation therapy (ADT) is the cornerstone of treatment for men with recurrent or metastatic prostate cancer (PCa). However, almost all patients eventually stop responding to ADT and develop castration-resistant prostate cancer (CRPC) [1]. Clinical diagnosis of CRPC is based on a significant increase in tumor burden or metastasis detected using computed tomography (CT) scans or magnetic resonance imaging (MRI) and/or rising serum prostate-specific antigen (PSA) levels [2]. Currently no reliable clinical or non-invasive imaging methods can specifically predict the development of CRPC, which is critical in guiding treatment decisions in men with advanced PCa. Early detection of emerging CRPC, prior to diagnosis by prevailing clinical parameters, could prompt expeditious initiation of next-line therapies to slow or halt progression of disease.

Genomic analyses of PCa cell lines and patient tumors have shown that androgen receptor (AR) is still active in some subtypes of CRPC [3,4] despite castration levels of testosterone and that its DNA binding profile changes [5]. Expression of AR-regulated genes including those involved in glucose uptake and glycolysis, glutaminolysis, and anabolic metabolism have been shown to be altered in CRPC [6]. Furthermore, AR binding sites found only in CRPC were enriched for MYC [5], which regulates glycolysis and glutaminolysis [7]. In subtypes of CRPC with little or no AR, other oncoproteins may induce similar metabolic changes [8]. Altogether, these genetic findings suggest that metabolism of glucose and glutamine is altered in CRPC.

Hyperpolarized (HP) ^{13}C MRI is a powerful technology that can image metabolic fluxes through key pathways associated with cancer progression and therapeutic response [9]. Preclinical HP ^{13}C MRI studies have shown that glycolytic activity, as measured by the rate of conversion of ^{13}C pyruvate to ^{13}C lactate (k_{PL}) and the pyruvate-to-lactate ratio, increases as PCa become more aggressive [10–12]. Additionally, HP ^{13}C MRI demonstrated an early reduction in k_{PL} after ADT in a metastatic lesion of a patient with PCa, preceding clinical response to therapy [13]. While initial preclinical and patient studies have focused on using FDA-approved HP [1- ^{13}C]pyruvate to investigate changes in glycolysis, clinical translation of HP probes that provide insight into other metabolic pathways, such as [2- ^{13}C]pyruvate and [5- ^{13}C]glutamine, is in progress.

Although HP ^{13}C MRI has shown potential to monitor metabolic changes that occur during progression of PCa and in response to therapy, its relevance to detection of CRPC remains speculative pending further definition of the metabolic phenotype of CRPC. Here, human PCa cell lines representative of androgen-dependent PCa (LNCaP) and CRPC (PC-3) were compared to identify metabolic pathways related to the castration-resistant phenotype. To more closely mimic the *in vivo* progression from androgen-dependence to CRPC, the transgenic adenocarcinoma of the mouse prostate (TRAMP) model was also studied [14]. Similar to human PCa, TRAMP tumors are initially androgen-dependent and progress to CRPC upon resistance to orchiectomy, which is equivalent to chemically-induced ADT [15,16]. The metabolism of [U- ^{13}C]glucose and [U- ^{13}C]glutamine in these models was evaluated by 1D and 2D high-field nuclear magnetic resonance (NMR) spectroscopy. Measurement of steady state levels and fractional enrichment of metabolites accompanied by ^{13}C isotopomer modeling, enzymatic assays, and determination of rates of oxygen consumption demonstrated an upregulation of glycolysis, TCA cycle, glutaminolysis and glutathione synthesis in CRPC. Finally, the ability of HP [1- ^{13}C]pyruvate NMR spectroscopy to distinguish PC-3 from LNCaP cells and androgen-dependent versus CRPC TRAMP tumors due to upregulated glycolysis was demonstrated.

2. Results

2.1. Differences in steady state levels of metabolites between androgen-dependent and castration-resistant PCa

To assess utilization of glucose and glutamine in androgen-dependent PCa (ADPC) versus CRPC, LNCaP and PC-3 cells were labeled with [U- ^{13}C]glucose or [U- ^{13}C]glutamine *in vitro*, and ADPC and CRPC TRAMP tumors were labeled *in vivo* in mice infused with [U- ^{13}C]glucose or [U- ^{13}C]glutamine. Soluble metabolites extracted from cells and tumors were analyzed by high-field NMR spectroscopy. Representative 1D ^1H spectra with water presaturation clearly highlight the differences in the metabolic flux of glucose and glutamine between the ADPC versus CRPC cell lines (Figure S1A and B) and TRAMP tumors (Figure 1A and B). Total and ^{13}C -labeled metabolite concentrations were quantified using ^{13}C -decoupled ^1H spectroscopy (Figure S2) and 2D ^1H - ^{13}C HSQC (Figure 1C and D and Figure S1C–D), respectively. Steady-state concentrations of metabolites in each of the cell lines and TRAMP tumors are listed in Supplementary Table S1. Levels of aspartate, glutamate, lactate, myo-inositol, phosphocholine, glycerophosphocholine, and total choline were significantly higher in castration-resistant PC-3 cells compared to androgen-dependent LNCaP cells, while levels of citrate, glucose, creatine and creatine phosphate were significantly lower in PC-3 compared to LNCaP cells. Similarly, levels of lactate were significantly higher, and citrate and creatine were significantly lower, in

CRPC compared to ADPC TRAMP tumors. A schematic of the metabolic pathways involving several of these metabolites is shown in **Figure 2**. Altogether, the results demonstrate the components of glycolysis (glucose, lactate), the TCA cycle (aspartate, citrate, glutamate), glutaminolysis (glutamate) and redox capacity (glutathione) are altered in CRPC compared to ADPC.

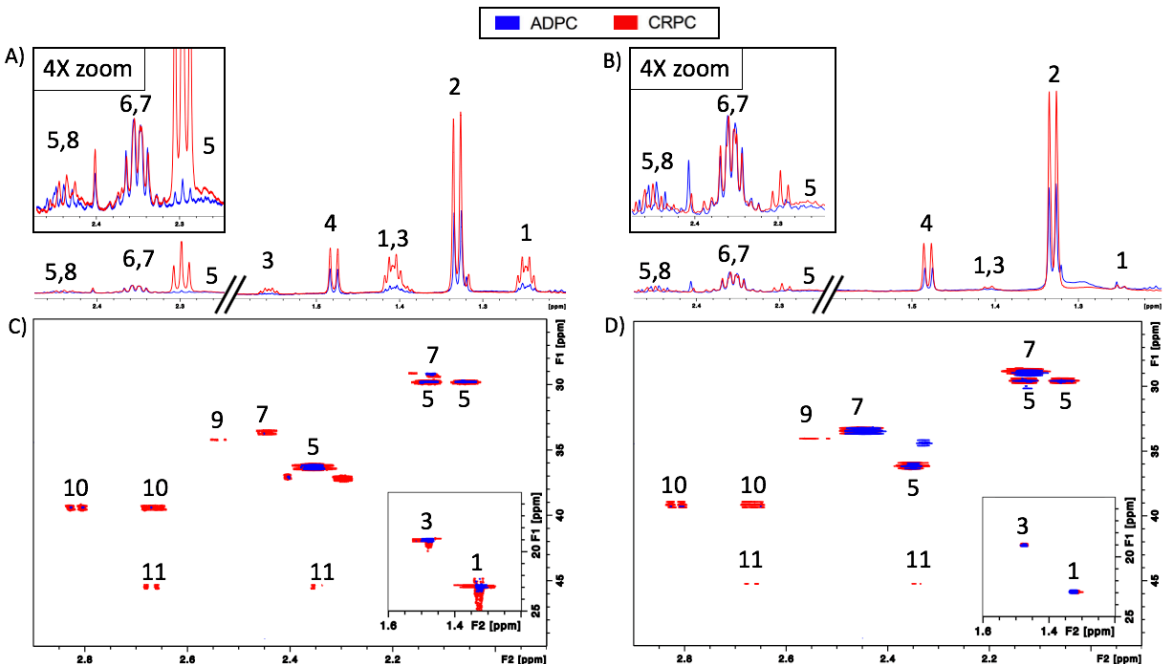
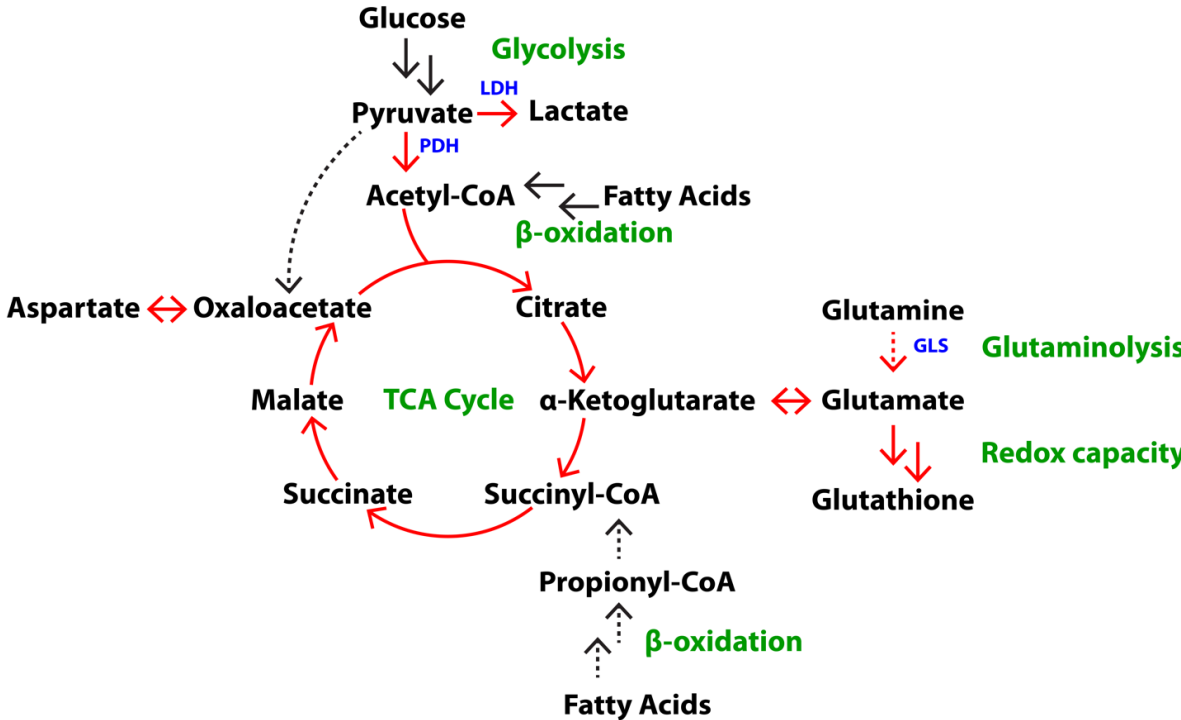


Figure 1. High-resolution NMR spectra of tumor extracts. Representative 1D ^1H spectra with water presaturation of ADPC (blue) and CRPC (red) TRAMP tumors labeled with **A**, $[\text{U-}^{13}\text{C}]$ glucose and **B**, $[\text{U-}^{13}\text{C}]$ glutamine. Zoomed region indicates glutamate C4 region with vertical scale increased by 4-fold. High resolution 2D $^1\text{H-}^{13}\text{C}$ HSQC of ADPC and CRPC TRAMP tumors labeled with **C**, $[\text{U-}^{13}\text{C}]$ glucose and **D**, $[\text{U-}^{13}\text{C}]$ glutamine. Metabolite peaks are labeled as follows: 1. ^{13}C -Lactate, 2. Lactate, 3. ^{13}C -Alanine, 4. Alanine, 5. ^{13}C -Glutamate, 6. Glutamate, 7. ^{13}C -Glutamine, 8. Glutamine, 9. ^{13}C -Glutathione, 10. ^{13}C -Aspartate, 11. ^{13}C -Malate.

Figure 2. Overview of metabolic pathways associated with glucose and glutamine



metabolism. Key metabolic pathways (green) and respective metabolites (black) and

enzymes (blue) are shown. Red arrows indicate pathways that are particularly relevant to distinguishing CRPC from ADPC, doubled arrows represent simplified multistep processes, and dashed lines indicate anaplerotic pathways of the TCA cycle.

2.2. Elevated glycolysis in CRPC versus ADPC

In addition to higher steady-state levels of lactate in PC-3 compared to LNCaP cells (Supplementary Table S1), the rate of lactate efflux from cells into the culture medium was also significantly higher for PC-3 compared to LNCaP cells (**Figure 3A**). Quantification of the fractional enrichment (FE) of lactate in ^{13}C -glucose labeling studies showed that PC-3 cells had significantly increased lactate FE compared to LNCaP cells (**Figure 3B**), indicating increased glucose flux through glycolysis (**Figure 2**). This finding was supported by the significantly elevated enzymatic activity of LDH, which converts pyruvate to lactate (**Figure 2**), in PC-3 compared to LNCaP cells (**Figure 3C**). The consumption rate of glucose, however, did not differ between the cell lines (**Figure 3D**). Together, these data indicate that glucose flux through glycolysis is upregulated in PC-3 relative to LNCaP cells.

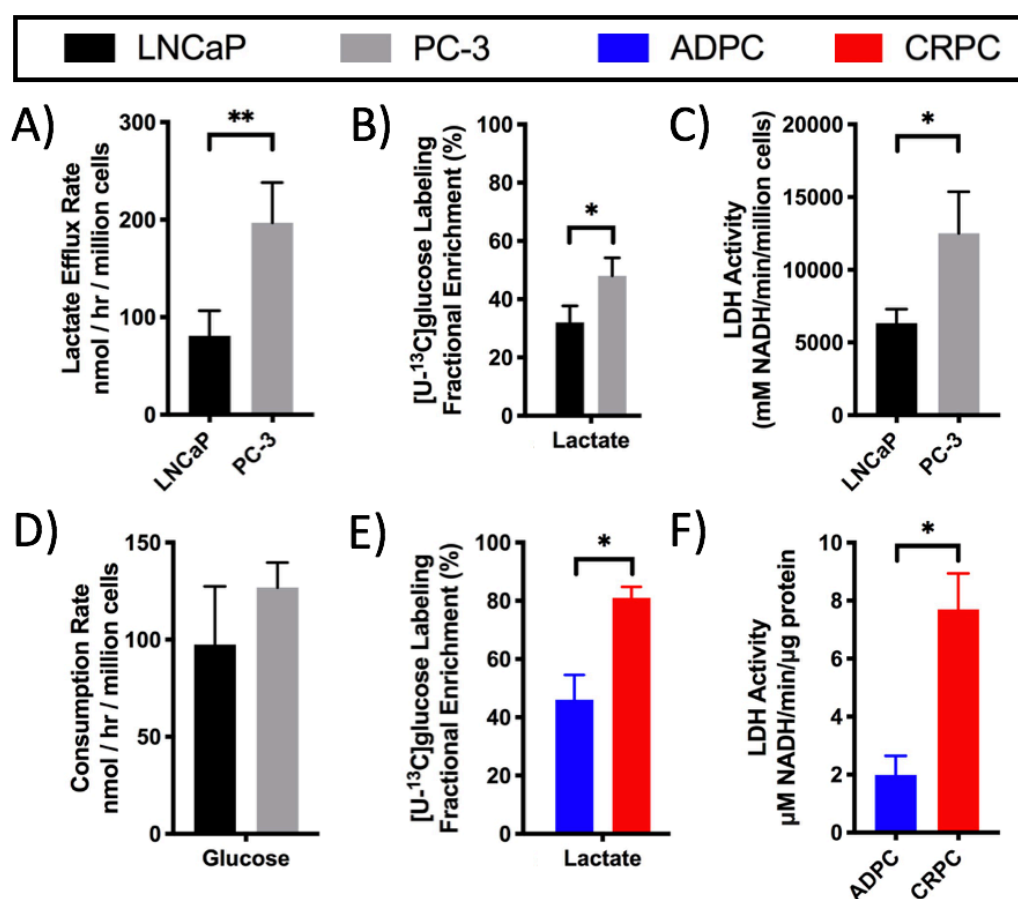


Figure 3. CRPC cells and tumors exhibit elevated glycolytic activity. **A**, Lactate efflux rate and **B**, steady state fractional enrichment of lactate were quantified in LNCaP and PC-3 cells labeled with [U- ^{13}C]glucose (N=3). **C**, Enzymatic activity of LDH in LNCaP and PC-3 cells was determined by biochemical assay of protein lysates (N=3). **D**, Steady-state consumption rates of [U- ^{13}C]glucose by LNCaP and PC-3 cells (N=3). **E**, Fractional enrichment of lactate and **F**, LDH activity were determined in ADPC and CRPC TRAMP tumors labeled with [U- ^{13}C]glucose (N=3).

The glycolytic alterations occurring in cell lines in vitro were recapitulated in vivo in the TRAMP model mimicking the development of CRPC in patients. In [U- ^{13}C]glucose-labeling studies, FE of lactate was significantly higher in CRPC compared to ADPC TRAMP tumors (**Figure 3E**), indicating increased flux of glucose through glycolysis as seen in PC-3 compared to LNCaP cells. Also similar

to the cell lines, LDH activity was higher in CRPC compared to ADPC TRAMP tumors (**Figure 3F**). In all, higher levels of steady-state lactate, increased FE of lactate in ^{13}C -glucose labeling studies, and elevated activity of LDH indicate that glycolysis is upregulated in human and murine models of CRPC compared to their androgen-dependent counterparts.

2.3. Increased utilization of pyruvate in the TCA cycle in CRPC versus ADPC

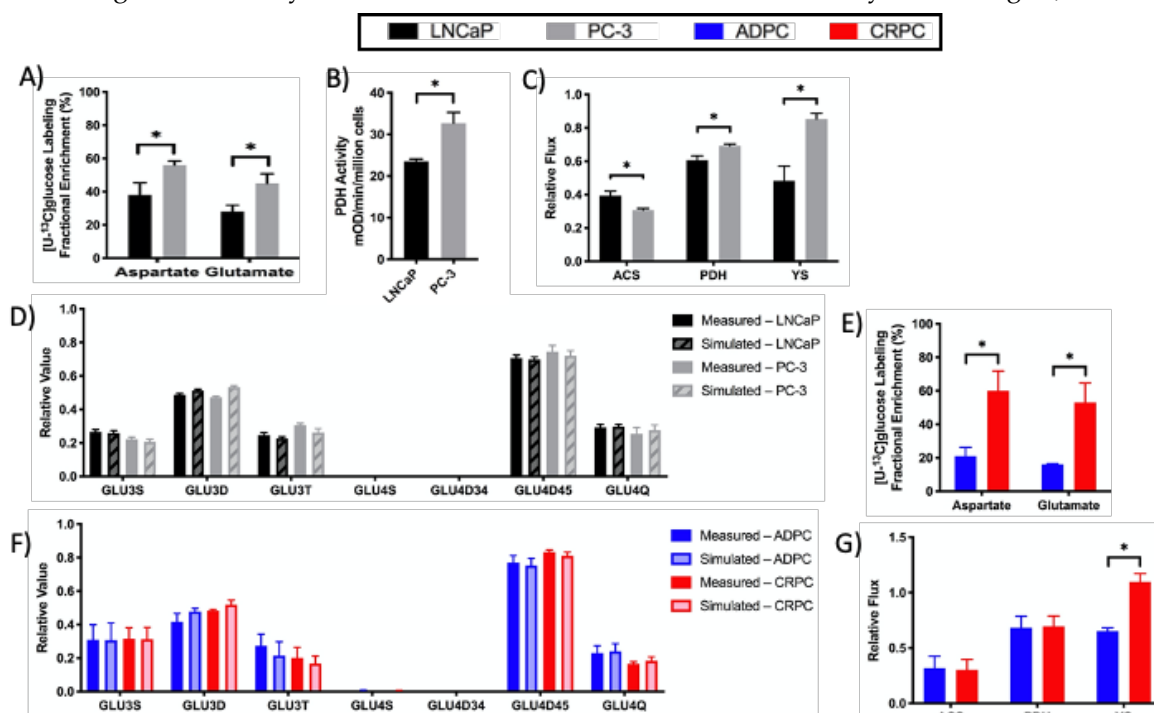
The TCA cycle is a central metabolic pathway that supports aerobic respiration, macromolecule synthesis, and redox balance. As shown in **Figure 2**, several different metabolites can fuel the TCA cycle, including pyruvate, glutamine, and fatty acids. Preferences in substrate utilization as well as the overall activity of the TCA cycle can be characterized by measuring the FE of aspartate and glutamate, both of which are in fast exchange with low-abundance intermediates of the TCA cycle [17]. In addition, the intermediates of the TCA cycle can be used to fuel macromolecule synthesis of carbohydrates, lipids, and proteins. The role of the TCA cycle in prostate metabolism is particularly interesting, since the healthy prostate gland is responsible for producing and secreting large amounts of citrate. This results in a truncated TCA cycle [18]. In PCa, citrate is oxidized to support energy production and the TCA cycle is restored. However, the metabolic activity of the TCA cycle in CRPC is unknown.

As described above (Supplementary Table S1), LNCaP compared to PC-3 cells had a significantly higher steady-state level of citrate and lower levels of aspartate and glutamate, which can inform on TCA cycle activity downstream of citrate (**Figure 2**). These findings suggest that LNCaP cells, similar to normal prostate, favor reduced TCA cycle activity in order to use intermediates of the TCA cycle for macromolecule synthesis, while PC-3 cells utilize a fully functional TCA cycle in order to maximize energy production. This differential utilization of the TCA cycle is further supported by the $[\text{U-}^{13}\text{C}]$ glucose-labeling results shown in **Figure 4A**, which revealed that PC-3 compared to LNCaP cells had significantly increased FE of aspartate and glutamate, suggesting increased glucose utilization through the TCA cycle in PC-3 cells (**Figure 2**). The enzymatic activity of pyruvate dehydrogenase (PDH), which converts pyruvate into acetyl-CoA (**Figure 2**), was also higher in PC-3 than LNCaP cells (**Figure 4B**). To corroborate these findings, ^{13}C isotopomer modeling using TCACALC was performed using the relative isotopomer ratios of glutamate derived from $[\text{U-}^{13}\text{C}]$ glucose in LNCaP and PC-3 cells. ^{13}C isotopomer modeling of $[\text{U-}^{13}\text{C}]$ glucose-labeled cell extracts indicated significantly upregulated PDH flux in PC-3 cells (**Figure 4C**). A good fit of the model was observed as reflected by the simulated isotopomer ratios of glutamate closely matching experimental values (**Figure 4D**). Furthermore, over 95% of the pyruvate is uniformly labeled, indicating that the majority of pyruvate entering the TCA cycle results from $[\text{U-}^{13}\text{C}]$ glucose. These studies demonstrated that PC-3 cells had more pyruvate entering the TCA cycle via pyruvate oxidation by PDH than other anaplerotic sources such as acetate (via increased ACS activity) and other fatty acid sources resulting from β -oxidation (**Figure 2**). Together, these results illustrate that glucose flux through the TCA cycle is upregulated in PC-3 cells whereas in LNCaP cells, although the uptake of glucose is similar, utilization of glucose for TCA cycle is reduced.

Similar to the castration-resistant versus androgen-dependent cell lines, CRPC TRAMP tumors exhibited increased FE of glutamate and aspartate compared to ADPC TRAMP tumors after labeling with $[\text{U-}^{13}\text{C}]$ glucose, suggesting increased pyruvate flux through TCA metabolism (**Figure 4E**). Altogether, increased utilization of glucose in the TCA cycle was characteristic of both human and murine models of CRPC compared to ADPC. Interestingly, ^{13}C isotopomer modeling of $[\text{U-}^{13}\text{C}]$ glucose-labeled ADPC and CRPC TRAMP tumors (**Figure 4F and G**) revealed no significant difference in PDH flux relative to ACS flux, which is also what we observed in biochemical assay (data not shown). This could indicate utilization of pyruvate via pyruvate carboxylase as an anaplerotic source in the TCA cycle thereby contributing to increased aspartate and glutamate

fractional enrichment as well (**Figure 4G**). Therefore, the increased activity through the TCA cycle observed in CRPC TRAMP tumors could be due to increased pyruvate carboxylase activity rather than primarily via PDH.

Figure 4. Pyruvate oxidation is increased in CRPC to fuel the TCA cycle. Flux of pyruvate through the TCA cycle was assessed in LNCaP and PC-3 cells by measuring **A**, the



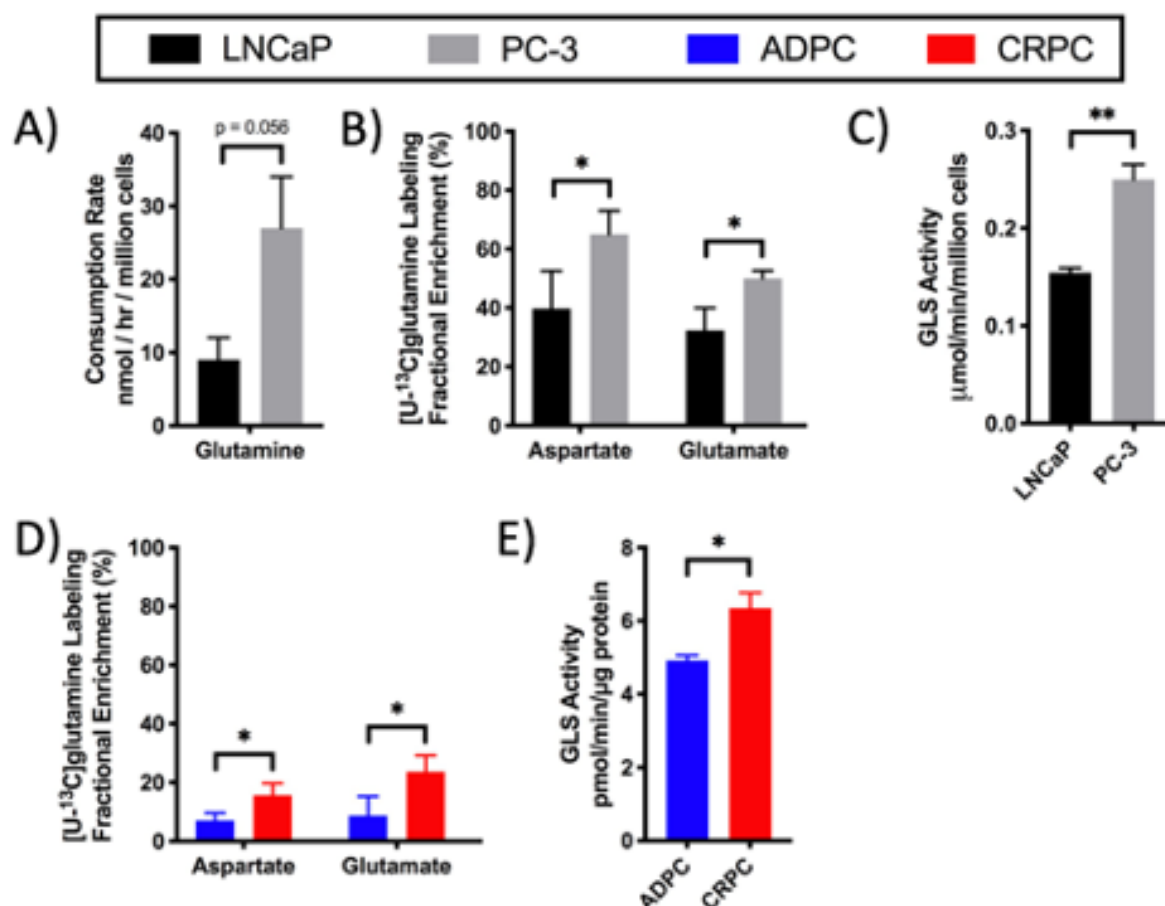
fractional enrichment of aspartate and glutamate in cells labeled with [U-¹³C]glucose and **B**, enzymatic activity of PDH. **C**, Calculated values for the relative flux of unlabeled acetate and other fatty acids into the TCA cycle (ACS), relative ¹³C-glucose flux into the TCA cycle (PDH), and the anaplerotic contribution relative to citrate synthase (YS) using TCACALC. **D**, ¹³C isotopomer modeling of [U-¹³C]glucose-labeled LNCaP and PC-3 cells using TCACALC showed measured and simulated relative ¹³C isotopomer areas of the glutamate C3 and C4 multiplets. Flux of pyruvate through the TCA cycle was determined in ADPC and CRPC TRAMP tumors by measuring **E**, fractional enrichment of aspartate and glutamate of tumors labeled with [U-¹³C]glucose. ¹³C isotopomer modeling of [U-¹³C]glucose-labeled ADPC and CRPC TRAMP tumors using TCACALC showed **F**, measured and simulated relative ¹³C isotopomer areas of the glutamate C3 and C4 multiplets and **G**, calculated values for the relative flux of ACS, PDH, and YS.

2.4. Glutamine utilization in the TCA cycle and glutaminolysis in CRPC versus ADPC

¹³C isotopomer modeling indicated that PC-3 cells had significantly higher relative anaplerotic flux (YS) compared to LNCaP cells (**Figure 4C**). To determine the anaplerotic fuel source, glutamine metabolism and its relative anaplerosis to the TCA cycle were assessed. As described above (Supplementary Table S1), PC-3 compared to LNCaP cells had significantly higher concentrations of steady-state glutamate, which could be generated directly from glutamine (**Figure 2**). Furthermore, [U-¹³C]glutamine-labeling studies showed that PC-3 cells consumed glutamine at a significantly higher rate than LNCaP cells (**Figure 5A**). PC-3 cells also exhibited increased glutamine flux through glutaminolysis and the TCA cycle based on the significantly elevated FE of aspartate and glutamate in PC-3 compared to LNCaP cells (**Figure 5B**). Correspondingly, PC-3 cells also had significantly increased activity of glutaminase (GLS), which converts glutamine to glutamate, compared to LNCaP

cells (**Figure 5C**), in accord with the significantly increased activity through glutaminolysis observed after $[U-^{13}C]$ glutamine labeling. In sum, these results indicate that glutaminolysis and its subsequent anaplerosis are upregulated in PC-3 compared to LNCaP cells.

Figure 5. Glutamine metabolism through glutaminolysis and the TCA cycle is enhanced in



CRPC. Glutamine metabolism was assessed in LNCaP and PC-3 cells based on **A**, consumption rate of $[U-^{13}C]$ glutamine, **B**, the fractional enrichment of aspartate and glutamate of cells labeled with $[U-^{13}C]$ glutamine and **C**, enzymatic activity of GLS. Flux through glutaminolysis and the TCA cycle was determined in ADPC and CRPC TRAMP tumors based on **D**, the fractional enrichment of aspartate and glutamate of tumors labeled with $[U-^{13}C]$ glutamine and **E**, enzymatic activity of GLS.

Glutaminolysis and glutamine anaplerosis into the TCA cycle were then assessed in the TRAMP model. While the steady-state concentrations of aspartate and glutamate were not significantly different between ADPC and CRPC TRAMP tumors (Supplementary Table S1), $[U-^{13}C]$ glutamine-labeling studies revealed that CRPC TRAMP tumors had significantly increased FE of aspartate and glutamate compared to ADPC tumors, demonstrating increased flux through glutaminolysis and increased anaplerosis into the TCA cycle (**Figure 5D**). The upregulation of glutaminolysis in CRPC tumors is further supported by significantly higher activity of GLS in CRPC versus ADPC TRAMP tumors (**Figure 5E**). Interestingly, the low fractional enrichment in TRAMP tumors shows that they are not as glutamine-reliant as LNCaP and PC-3 cells. Altogether, these results indicate that upregulation of glutaminolysis and glutamate anaplerosis into the TCA cycle are characteristic of castration-resistant versus androgen-dependent PCa.

2.5. Differential substrate utilization into oxidative phosphorylation (OXPHOS) by LNCaP and PC-3 cells

To better understand the relative utilization of glucose, long-chain fatty acids, and glutamine in the TCA cycle by ADPC versus CRPC, the oxygen consumption rate (OCR) was measured using the Oxygraph+ and substrate preferences were assessed using the Seahorse XFe24 Extracellular Flux Analyzer. LNCaP cells displayed higher basal OCR compared to PC-3 cells (Supplementary Figure S3A), suggesting that LNCaP cells could have higher oxidative phosphorylation of glucose and beta-oxidation compared to PC-3 cells. This is in line with the observation of higher contribution of OXPHOS of glucose in LNCaP cells (Supplementary Figure S3B), resulting in increased glucose conversion to glutamate (via the TCA cycle, Supplementary Figure S3E) and also supported by the increased ACS flux from TCACalc modeling data (**Figure 4C**). This highlights the contribution of other substrates (besides glucose and glutamine) to the TCA activity in LNCaP cells. While glucose was the primary fuel source for OXPHOS in both cell lines when measured using the Seahorse Mito Fuel Flex kit, PC-3 cells had significantly higher net anaplerosis than LNCaP cells (Supplementary Figure S3B). PC-3 cells relied more on glutamine and long-chain fatty acids as anaplerotic fuel sources compared to LNCaP cells. Alternatively, LNCaP cells relied more on other anaplerotic contributors to mitochondrial respiration than PC-3 cells. These other sources include anaplerotic substrates such as short and medium chain fatty acids (e.g., propionate, butyrate, octanoate), amino acids that enter through oxaloacetate (e.g., aspartate, asparagine), and branched-chain amino acids that enter through succinyl-CoA (e.g., leucine, isoleucine, and valine) [19].

Cellular bioenergetics were also assessed to understand the differential upregulation of glycolysis and the TCA cycle to meet the energy needs of LNCaP and PC-3 cells. The total ATP content was significantly higher in PC-3 than LNCaP cells (Supplementary Figure S3C), suggesting that PC-3 cells have higher energy capacity, supporting the observed upregulation of both glycolysis and the TCA cycle in PC-3 cells. Furthermore, PC-3 cells had a significantly lower NAD⁺/NADH ratio compared to LNCaP cells (Supplementary Figure S3D), which is consistent with the significantly upregulated glycolysis in PC-3 cells. This is further supported by the mass balance analysis of ¹³C-glucose-labeled metabolites, which demonstrated that PC-3 cells utilized the majority of glucose consumed to produce lactate (Supplementary Figure S3E).

2.6. Increased redox capacity in CRPC versus ADPC

The significantly higher level of steady-state glutathione in PC-3 compared to LNCaP cells (Supplementary Table S1) suggests a difference in redox capacity between the two types of cells. Furthermore, [U-¹³C]glutamine-labeling studies revealed that PC-3 cells had significantly increased FE of glutathione, quantified using 2D 1H-1H TOCSY (Supplementary Figure S1E), compared to LNCaP cells (**Figure 6A**), suggesting increased glutamine flux through glutathione synthesis to support redox balance (**Figure 2**). This correlates with the significantly increased ratio of reduced glutathione relative to total glutathione (**Figure 6B**) and increased NADP⁺/NADPH ratio (**Figure 6C**) in PC-3 compared to LNCaP cells. Taken together, these results suggest that PC-3 cells have increased glutathione redox capacity compared to LNCaP cells.

[U-¹³C]glutamine-labeling studies also showed that CRPC TRAMP tumors had significantly elevated FE of glutathione compared to ADPC tumors (**Figure 6D**), suggesting increased glutathione synthesis to support redox balance. The biochemical assay revealed a significantly higher total glutathione pool in CRPC relative to ADPC tumors (**Figure 6E**). Overall, these results suggest that CRPC has higher glutathione redox potential compared to ADPC.

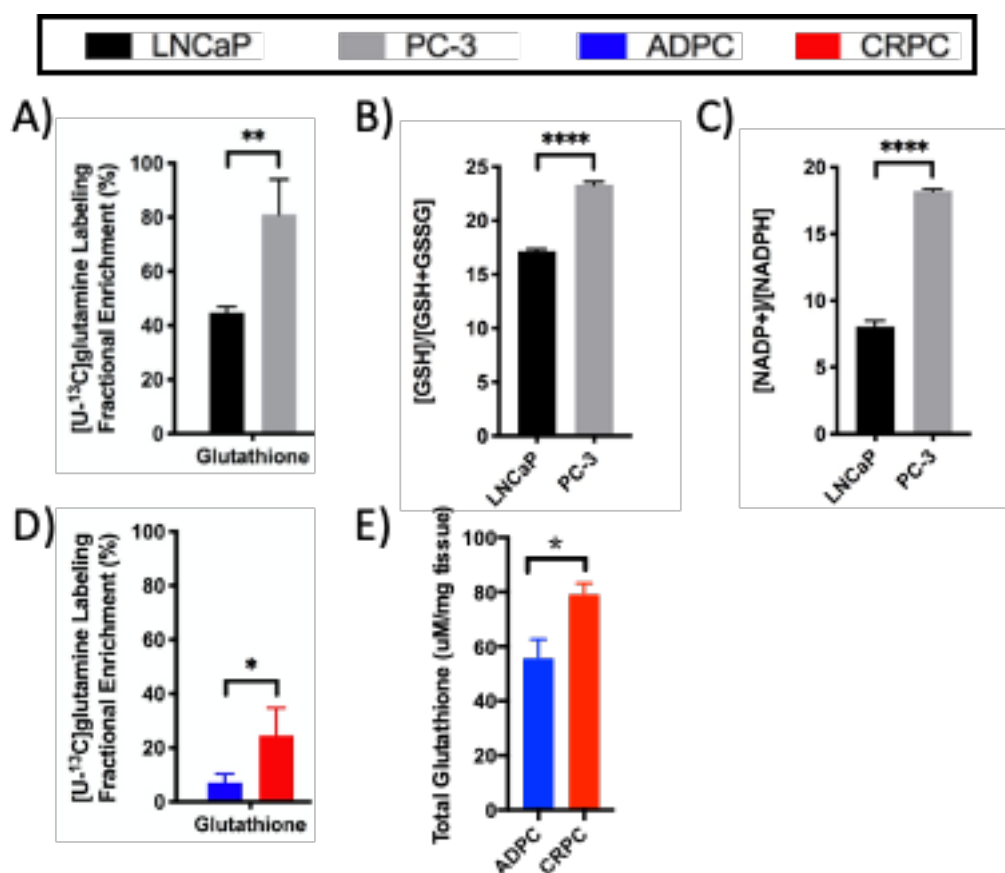


Figure 6. Glutathione redox capacity is upregulated in CRPC. Glutathione redox capacity was assessed in LNCaP and PC-3 cells based on **A**, fractional enrichment of glutathione in cells labeled with [U-¹³C]glutamine, **B**, ratio of reduced glutathione (GSH) relative to total glutathione (GSH+GSSG), and **C**, intracellular NADP⁺/NADPH. Glutathione redox balance was assessed in ADPC and CRPC TRAMP tumors based on **D**, fractional enrichment of glutathione in tumors labeled with [U-¹³C]glutamine and **E**, total glutathione.

2.7. Elevated glycolysis observed in CRPC vs ADPC using HP [1-¹³C]pyruvate

Metabolic differences identified between ADPC and CRPC led us to assess the ability of HP [1-¹³C]pyruvate NMR spectroscopy to measure increased glycolysis in PC-3 over LNCaP cells. A schematic of the experimental setup is shown in **Figure 7A**. Dynamic 1D ¹³C NMR spectral data were acquired from slurries of live cells (**Figure 7B-C**) and peak areas were integrated (**Figure 7D**). PC-3 cells had a significantly higher pyruvate-to-lactate conversion rate, k_{PL} , than LNCaP cells, consistent with the upregulation of glycolysis as described by the metabolomics findings (**Figure 7E**). This demonstrates that HP [1-¹³C]pyruvate can be used to non-invasively assess glycolytic capacity.

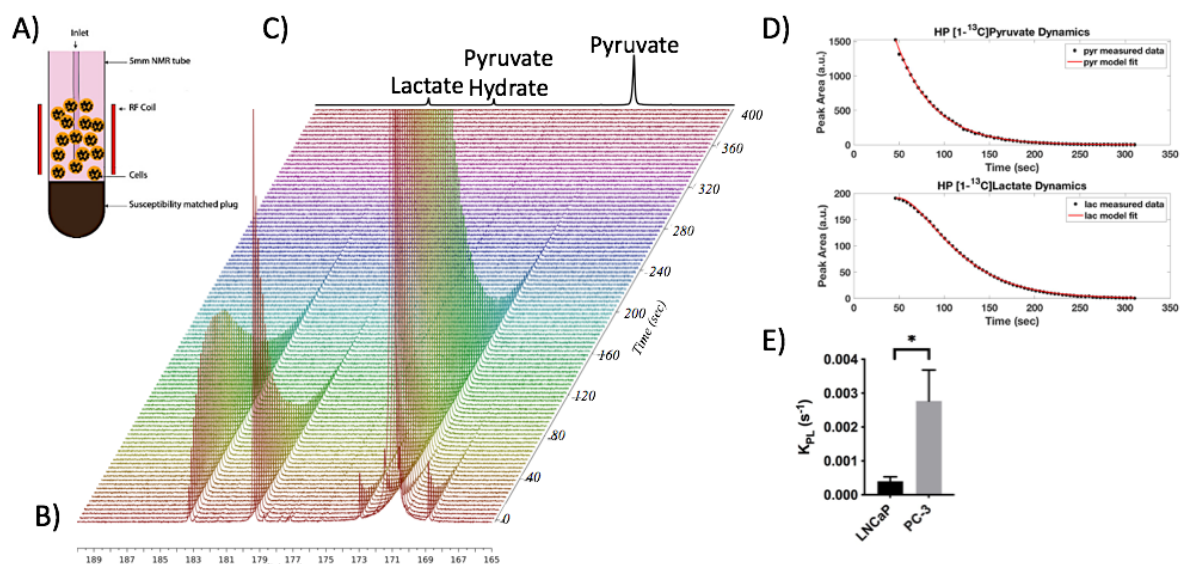


Figure 7. Increased glycolytic activity of CRPC is measured in real time using HP $[1-^{13}\text{C}]$ pyruvate. **A**, Schematic of the cell slurry experimental design shows the cells resuspended in culture medium in a 5-mm NMR tube. Representative **B**, dynamic and **C**, summed HP ^{13}C spectra show the lactate signal that arises from $[1-^{13}\text{C}]$ pyruvate. **D**, Kinetics of peak-fitted pyruvate and lactate show robust production of lactate. **E**, Pyruvate to lactate conversion rate (k_{PL}) ($p < 0.05$, $n = 4$).

Metabolism of HP $[1-^{13}\text{C}]$ pyruvate was assessed *in vivo* in the orchiectomy-driven TRAMP model of CRPC using 3D chemical shift imaging (CSI) (**Figure 8A**). There was no significant difference in the baseline (pre-orchiectomy) volumes between the tumors that exhibited sensitivity to androgen deprivation versus those exhibited castration-resistance post-orchiectomy (Supplementary Figure S4A). Similarly, no significant difference in Lac/Pyr ratio was observed between the two types of tumors prior to orchiectomy (Supplementary Figure S4B). Representative images and spectrum from both the ADPC and CRPC tumors at baseline and subsequent follow-up are shown in **Figure 8A**. On average the mice in the ADPC group survived (46 ± 10 days) twice as long as those in the CRPC group (20 ± 7 days). Immunohistochemical staining of Ki67 (**Figure 8B**) clearly shows the increased proliferation in CRPC tumors (greater than 90% compared to ~1% staining in ADPC tumors, Supplementary Figure S4C). There was a mean reduction in tumor volume observed in the ADPC group compared to a significant doubling of tumor volume in the CRPC group after castration (**Figure 8C**). Subsequently the changes observed in the HP Lac/Pyr ratio followed the similar significant trend of decreased glycolysis in androgen-dependent tumors relative to the CRPC tumors (**Figure 8D**). This is in accord with our *ex vivo* metabolic findings using stable isotope tracers and provides support for using the Lac/Pyr ratio as a marker of therapeutic resistance to ADT.

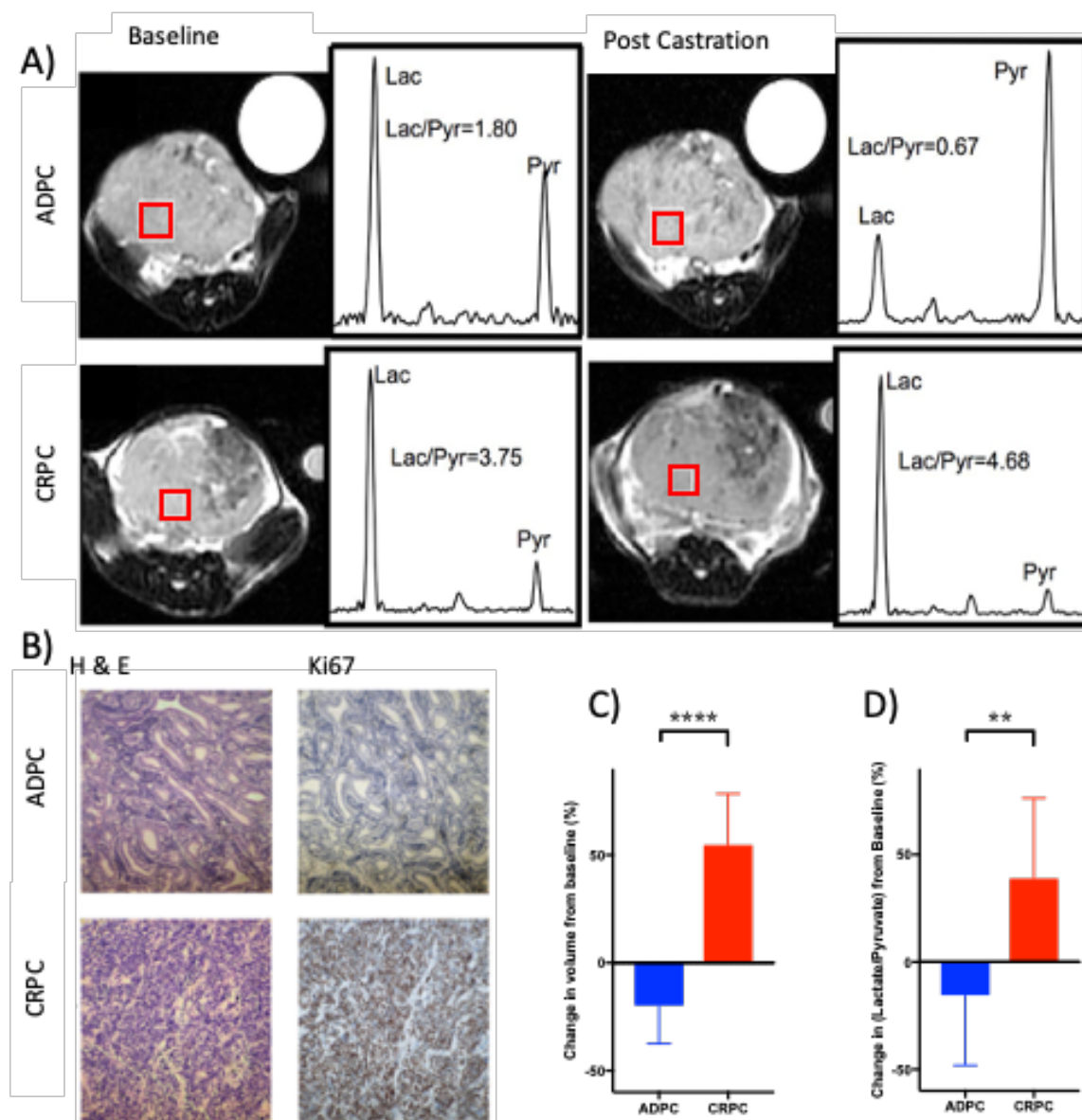


Figure 8. In vivo metabolism of hyperpolarized $[1-^{13}\text{C}]$ pyruvate in a treatment-driven TRAMP model. **A**, Representative T2-weighted proton images of ADPC (top row) and CRPC (bottom row) TRAMP mouse at baseline (left column) and 5 days post-castration (right column). The spectrum from a voxel (red square) is shown on the right of each image of the hyperpolarized lactate (Lac) and pyruvate (Pyr) and the calculated ratio (Lac/Pyr). **B**, Representative images of immunohistochemical staining of ADPC (top row) and CRPC (bottom row) of hematoxylin and eosin (H&E), Ki67 (indicator of proliferation) and caspase-3 (indicator of apoptosis). **C**, Change in volume and **D**, hyperpolarized Lac/Pyr of TRAMP tumors from baseline to post-orchietomy in ADPC (n=11) and CRPC (n=7) mice.

3. Discussion

In this study, metabolic changes associated with the development of CRPC were characterized in human PCa cell lines and in the TRAMP murine model of progression of androgen-dependence to castration-resistance. Steady-state metabolite concentrations and FE were measured in $[U-^{13}\text{C}]$ glucose- and $[U-^{13}\text{C}]$ glutamine-labeled cells and tumors using high-resolution 1D and 2D NMR techniques, which revealed that CRPC exhibited increased flux through glycolysis, TCA metabolism

of pyruvate and glutamine, glutaminolysis, and glutathione synthesis in both the human and murine models. This suggests that CRPC tumors have increased bioenergetic and biosynthetic demands to support castration-resistant growth.

Our finding that PC-3 cells had increased lactate production and export compared to LNCaP cells is consistent with previous findings [20]. We observed that LNCaP and PC-3 cells have similarly high rates of glucose consumption, in accordance with other studies that showed upregulation of glycolysis despite no significant difference in glucose consumption in castration-resistant cell and xenograft models [20-23].

Interestingly, PC-3 cells had lower OCR than LNCaP cells despite having higher PDH activity. This matches published studies which concluded that LNCaP cells rely mainly on OXPHOS for ATP generation while PC-3 cells rely mainly on glycolysis [24,25]. Previous studies have shown that AR signaling promotes a truncated TCA cycle by stimulating anabolism of glucose to citrate, resulting in significantly higher citrate levels but no change in succinate levels [6]. This is supported by the significantly higher steady-state citrate concentration but no significant difference in succinate in androgen-dependent LNCaP compared to castration-resistant PC-3 as shown in Supplementary Table S1.

One key metabolic characteristic of CRPC is glutamine addiction. Increased glutamine consumption and subsequent utilization in glutaminolysis, glutamine anaplerosis into the TCA cycle, and glutathione synthesis observed in our study of CRPC cells and tumors has previously been reported [26]. In addition to supporting bioenergetic and biosynthetic needs, increased glutaminolysis is required for activation of the mTORC1 signaling pathway, which upregulates metabolism of glucose, glutamine and other amino acids, and lipids [27]. Interestingly, increased glutaminolysis also supported upregulation of glutathione-dependent redox pathways in both castration-resistant PC-3 cells and the treatment-emergent CRPC TRAMP model. Several studies have shown that CRPC has higher redox capacity based on increased expression of glutathione-related antioxidant genes such as glutathione peroxidase (GPX2) and glutathione synthase (GSS) [28,29]. This may be a critical step in the development of CRPC since glutathione prevents apoptosis by neutralizing free radicals and reactive oxygen species that would otherwise cause oxidative damage to DNA, proteins, and lipids [30].

To our knowledge, we provide the first analysis of metabolic changes that occur as the TRAMP murine model of PCa progresses from androgen-dependence to CRPC after ADT. The TRAMP model is considered to be highly representative of human PCa [31]. We previously described the role of lactate metabolism in the initial stages of development of cancer in the prostates of TRAMP mice. Dual agent HP MRI with ^{13}C -pyruvate and ^{13}C -urea demonstrated an increased Warburg effect, with associated increase in activity of LDH, in high-grade tumors compared to low-grade tumors and the normal prostate [32]. In a triple-transgenic mouse model of PCa, knockdown of *LDHA* slowed primary tumor growth and development of metastases, implicating LDH activity and lactate production in progression of PCa [32].

PC-3 cells and CRPC TRAMP tumors are representative of an androgen-independent subtype of CRPC that lacks expression of AR but is not small cell neuroendocrine cancer (AR-/NE-) [33]. Nevertheless, many metabolic features of these castration-resistant models are similar to CRPC that retains expression of AR and/or AR splice variants. In the absence of AR, other factors may induce a similar metabolic transition. For instance, Connelly et al. reported that Foxa2 activated transcription of a subset of AR target genes in AR-negative CRPC TRAMP tumors [34]. Bluemn et al. found that elevated activity of FGF and MAPK pathways bypassed dependence on AR in AR-negative PCa [8]. Nevertheless, other studies suggest metabolic differences in AR-driven CRPC versus AR-negative CRPC. Bader et al. reported findings from an isotopomeric metabolic flux analysis that showed that AR-positive PCa had elevated expression of mitochondrial pyruvate carrier (MPC2), which facilitates import of cytosolic glucose-derived pyruvate into the mitochondrial matrix for subsequent metabolism into the TCA cycle, compared to AR-negative PCa [35]. Furthermore, the authors showed that AR-negative PCa models that lack MPC expression had increased glutamine uptake to support

glutamine anaplerosis into the TCA cycle, while androgen-dependent PCa relied on glucose-derived pyruvate rather than glutamine as the major carbon source of the TCA cycle.

Finally, we demonstrated that PC-3 cells converted HP ^{13}C -pyruvate to lactate at a higher rate than LNCaP cells, consistent with results from Zacharias et al. who examined PC-3 xenografts using HP ^{13}C -pyruvate MRI [36]. However, recent work by van Heijster et al. [37] did not reveal a significant difference using the same cell lines, which could be attributed to the much higher concentration of hyperpolarized pyruvate used in our study. Further, we have shown in an inducible model of castration-resistance that ADPC tumors are much less glycolytic than CRPC tumors and can be monitored non-invasively using HP $[1-^{13}\text{C}]$ pyruvate. HP ^{13}C -pyruvate MRI is FDA-approved as an IND for PCa and undergoing clinical trials in a number of centers around the world for diverse applications, including predicting aggressiveness of primary PCa (NCT02526368) and response to therapy (NCT02911467) [38]. Of note, HP ^{13}C -pyruvate MRI showed reduction in conversion of pyruvate to lactate as an early response to ADT that associated with therapeutic efficacy [13]. Our studies further suggest that HP ^{13}C -pyruvate MRI could be used to monitor conversion of ADPC to CRPC in patients undergoing ADT, detecting increased glycolysis perhaps earlier than current clinical parameters diagnose CRPC. Our studies also indicate that other HP probes that are under development, including $[2-^{13}\text{C}]$ pyruvate to assess TCA metabolism, $[5-^{13}\text{C}]$ glutamine to assess glutaminolysis, and $[1-^{13}\text{C}]$ dehydroascorbate to assess glutathione redox potential, could be developed as non-invasive imaging biomarkers of CRPC.

In summary, our findings using human and murine models are consistent with the hypothesis that castration-resistant growth alters several metabolic pathways, including glycolysis, glutaminolysis, TCA metabolism, and glutathione redox capacity. These pathways may provide biomarkers to predict the development of clinical CRPC or candidate drug targets for the treatment of CRPC in patients.

4. Materials and Methods

Cell culture, ^{13}C -labeling and extraction of metabolites

LNCaP (clone FGC, ATCC CRL-1740) and PC-3 (ATCC CRL-1435) cells were obtained from American Type Culture Collection (ATCC) and grown to 70–80% confluency in a 37°C incubator with 95% air/5% CO_2 . For metabolic profiling, cells were cultured in RPMI-1640 medium (LNCaP) or Ham's F-12K medium (PC-3) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin for 6 hours. For ^{13}C -glucose labeling, media were replaced with glucose and glutamine free RPMI-1640 supplemented with 25 mM $[\text{U}-^{13}\text{C}]$ glucose, 10 mM glutamine, 10% FBS, and 1% penicillin-streptomycin for 6 hours. For ^{13}C -glutamine labeling, media were replaced with glucose and glutamine free RPMI-1640 supplemented with 10 mM $[\text{U}-^{13}\text{C}]$ glutamine, 25 mM glucose, 10% FBS, and 1% penicillin-streptomycin for 24 hours. Culture media were collected at baseline and after labeling to quantify glucose consumption rate, glutamine consumption rate, and lactate export rate. Prior to extraction of metabolites, cells were rinsed with cold phosphate-buffered saline (PBS). Cellular metabolism was quenched by direct addition of cold methanol, and intracellular metabolites were extracted using cold 1:1:1 methanol:water:chloroform [39]. The aqueous fraction was isolated, lyophilized, and resuspended in 600 μL of D_2O with TSP for NMR analysis.

Generation of androgen-dependent and CRPC TRAMP tumors, ^{13}C -labeling and extraction of metabolites

Adult TRAMP mice were obtained from Roswell Park Cancer Institute. Mice with a solid tumor mass between 0.1 – 1 cc underwent orchiectomy. Tumor volume was monitored by T_2 -weighted spin echo MRI and $<25\%$ increase ~ 5 days (± 2 days) post-orchiectomy was defined as androgen-dependent prostate cancer (ADPC) while $>35\%$ increase was defined as CRPC. Tumor volume changes are depicted in Supplementary Figure S5 and are similar to the cohort of mice that underwent hyperpolarized imaging (Figure 8C). Metabolic labeling of TRAMP tumors was achieved by injecting either 80 μL of 25% wt/vol $[\text{U}-^{13}\text{C}]$ glucose or 200 μL of 35.73 mg/mL $[\text{U}-^{13}\text{C}]$ glutamine into the tail vein every 15 minutes for a total labeling time of 45 minutes [40]. To minimize the effects

of stress and anesthesia on metabolism, mice were briefly anesthetized using isoflurane for 2–3 minutes to perform ^{13}C -injection, then allowed to wake between injections. Tissue was collected immediately upon euthanasia and flash-frozen in liquid nitrogen for metabolomic analysis.

For extraction of metabolites, frozen tissue was homogenized using a Tissuelyser LT in 400 μL of cold methanol at 4°C . Intracellular metabolites were extracted using cold 1:1:1 methanol:water:chloroform [39]. The aqueous fraction was isolated, lyophilized, and resuspended in 400 μL of D_2O for NMR analysis. For aqueous samples, an external standard of TSP and $[2-^{13}\text{C}]\text{glycine}$ in a 1.5-mm NMR tube was used for quantification.

NMR acquisition and quantification

NMR spectra were acquired on an 800 MHz Bruker AvanceI equipped with a 5-mm triple resonance TXI cryoprobe. High-resolution 1D ^1H presaturation spectra were acquired with the following parameters: 90° flip angle, 32k points, 15 ppm sweep width, 12 second water presaturation, 1.4 second acquisition time, 13.4 second repetition time, 64 scans, 14 minute scan time. ^{13}C -decoupled ^1H water presaturation spectra were acquired with the following parameters: 90° flip angle, 24k points, 10 ppm sweep width, 12 second water presaturation, 0.5 second acquisition time, 12.5 second repetition time, 32 scans, 6 minute scan time. Adiabatic decoupling was applied during acquisition using a CHIRP pulse with 54 kHz sweep width (equivalent to 200 ppm @ 800 MHz), and shaped pulse power level of +2 dB with respect to the power level determined for ^{13}C -GARP. Phase-sensitive 2D ^1H - ^{13}C HSQC was used with the following parameters: 2048x4096 points, 6x120 ppm sweep width, 1.8 second repetition time, 0.297 seconds acquisition, 2 averages, $J_{\text{CH}} = 135$ Hz (average J_{CH} of 127, 130, and 145 Hz for glutamate C2, C3, and C4), 4 hour scan time. ^{13}C decoupling was not applied during acquisition in order to resolve ^{13}C isotopomers. Data were zero-filled in both dimensions to a final digital resolution of 2.9 Hz/point in the F2 dimension. 2D ^1H - ^1H TOCSY with water presaturation was used with the following parameters: 4096x512 points, 12x12 ppm sweep width, 2.2 second repetition time, 0.239 seconds acquisition time, 8 averages, 60 millisecond mixing time, 2.75 hour scan time. Data were zero-filled in both dimensions to a final digital resolution of 9.4 Hertz/point in the F2 dimension.

Targeted metabolic profiling was performed using Chenomx (version 8.1) as previously described [41]. 1D datasets were zero-padded by a factor of 2, apodized with a 0.5 Hz exponential filter, and manually phased and baseline corrected. For cells, intracellular and extracellular metabolite concentrations were quantified from ^1H water presaturation 1D spectra of unlabeled cell extracts and culture media, respectively. For tissues, total metabolite concentrations were quantified from ^{13}C -decoupled ^1H water presaturation 1D spectra of ^{13}C -labeled tissue extracts.

Fractional enrichment (FE) of key metabolites was quantified using a combination of 1D and 2D NMR methods, where $\text{FE} = \frac{[^{13}\text{C}\text{-labeled metabolite}]_{\text{HSQC}}}{[\text{total metabolite}]_{[13\text{C}]\text{H}}}$. The total metabolite concentration was quantified from ^{13}C -decoupled ^1H spectra by manually fitting peaks of interest using a Lorentzian-Gaussian shape using ACD/1D NMR Processor (version 9). 2D NMR datasets were zero-padded by a factor of 2, manually phased, and peak volumes were integrated using TopSpin (version 3.5). The concentration of ^{13}C -labeled metabolites was quantified using ^1H - ^{13}C HSQC by integrating the volumes of the cross-peaks and correcting for J_{CH} filtering, inversion efficiency, and number of equivalent protons, using Equation 1, adapted from Heikkinen et al. [42]:

$$\text{Moles}_{\text{Met}} = \frac{\text{Vol}_{\text{Met}}}{\text{Vol}_{\text{Gly}}} * \frac{N_{1\text{H},\text{Gly}}}{N_{1\text{H},\text{Met}}} * \frac{1}{\sin^2\left(\frac{\pi J_{\text{CH},\text{Met}}}{2J_{\text{CH},\text{Gly}}}\right)} * \frac{(IE_{v,\text{Gly}})^2}{(IE_{v,\text{Met}})^2} * \text{Moles}_{\text{Gly}} \quad (\text{Equation 1})$$

where Vol_{Met} and Vol_{Ref} are the peak volumes in the HSQC spectrum for the metabolite of interest (Met) and the $[2-^{13}\text{C}]\text{glycine}$ (Gly), respectively; $N_{1\text{H},\text{Met}}$ and $N_{1\text{H},\text{Gly}}$ are the number of protons for Met and $[2-^{13}\text{C}]\text{glycine}$; $J_{\text{CH},\text{Met}}$ and $J_{\text{CH},\text{Gly}}$ are the J-coupling in hertz for Met and $[2-^{13}\text{C}]\text{glycine}$; $IE_{v,\text{Met}}$ is the inversion efficiency of at the resonance frequency (ν) of Met. The J_{CH} coupling constants of key metabolites are provided in Supplementary Table S1. Glutathione FE from $[U-^{13}\text{C}]\text{glutamine}$ labeling studies was quantified using ^1H - ^1H TOCSY by integrating the peak volumes of the ^{13}C -satellites and the central unlabeled peak. A peak table is provided for quantification of metabolites using ^1H - ^{13}C HSQC (Supplementary Table S2) and ^1H - ^1H TOCSY (Supplementary Table S3).

¹³C isotopomer modeling

For [¹³C]glucose-labeled cell extracts, relative peak volumes of glutamate C3 and glutamate C4 isotopomer multiplets were quantified from high-resolution 2D ¹H-¹³C HSQC spectra using Topspin (version 4.0.6). Glutamate C2 was not included since it has the lowest signal to noise ratio and its doublets (2D12 and 2D23) were not fully resolved. Data were then analyzed using TCACALC [43]. Model parameters were optimized using non-linear least squares. Using nomenclature adopted by TCACALC, the parameters examined include relative fluxes through acetyl-CoA synthase (ACS), pyruvate dehydrogenase (PDH), pyruvate carboxylase (YPC), and unidirectional anaplerotic flux of substrates leading to succinyl-CoA relative to citrate synthase (YS). All flux rates are normalized to a citrate synthase (CS) flux of 1, which is equivalent to the TCA cycle flux. Here, the flux through CS is modeled as the sum ACS flux of acetate and other fatty acids and relative ¹³C-glucose flux into the TCA cycle (PDH) (i.e., PDH + ACS = 1). The isotopomer model was validated using ANOVA with the following metrics: (1) statistical fit of individual parameters (p-value < 0.05), (2) statistical fit of the overall pathway model [F-value > 100, p(F=0) < 0.0001], and (3) comparison of measured and simulated relative isotopomer areas. The best fit model included the following parameters: ACS, PDH, YS, isotopic enrichment of the ¹³C-tracer, unlabeled “fatty acids” pool to represent all metabolites that convert to acetyl-CoA other than glucose (such as acetate and other ketone bodies), and unlabeled “anaplerotic substrate” to represent all metabolites that enter the TCA cycle through other intermediates of the TCA cycle.

Enzyme activity assays

To measure lactate dehydrogenase (LDH) activity, cells and tissues were homogenized using a TissueLyser LT in cell lysis buffer (Cell Signaling) and LDH activity was measured spectrophotometrically by quantifying the linear decrease in NADH absorbance at varying pyruvate concentrations at 339 nm using a microplate reader. The maximum velocity (v_{max}) and the Michaelis–Menten constant (K_m) were estimated using the Lineweaver–Burk plot.

To measure PDH activity, cells and tissues were homogenized using a Dounce homogenizer in PBS containing protease inhibitor cocktail (Abcam) and 20 mM NaF to preserve endogenous PDH phosphorylation state and activity. PDH activity was measured spectrophotometrically using the PDH enzyme activity microplate assay kit (Abcam) according to manufacturer’s instructions.

To measure GLS activity, cells and tissues were homogenized using a TissueLyser LT in lysis buffer provided by the PicoProbe™ glutaminase activity assay kit (Biovision). GLS activity was measured fluorometrically at Ex/Em 535nm/587nm with a glutamic acid standard curve using a microplate reader.

ATP quantification

ATP content of cell lysates was measured using the CellTiter-Glo® luminescent cell viability assay kit (Promega) according to manufacturer’s instructions. Luminescence of the luciferase reaction with ATP was measured using a Veritas luminometer (Thermo Fisher).

NAD/NADH and NADP/NADPH assays

NAD/NADH ratio of cell lysates was measured spectrophotometrically using the NAD/NADH assay kit (Biovision) according to manufacturer’s instructions. Cells were homogenized in lysis buffer provided by the kit using a TissueLyser LT. NAD, NADH, and their ratio were determined by measuring the reaction of reporter dye with NADH at 450 nm using a microplate reader.

NADP/NADPH ratio of cell lysates was measured spectrophotometrically using the NADP/NADPH assay kit (Abcam) according to manufacturer’s instructions. Cells were homogenized in lysis buffer provided by the kit using a TissueLyser LT. NADP, NADPH, and their ratio were determined by monitoring NADP formation fluorometrically at Ex/Em 540nm/590nm using the Tecan Infinite (Thermo Fisher) microplate reader.

Glutathione assay

Reduced glutathione (GSH) and total glutathione of cell lysates were measured spectrophotometrically using the glutathione colorimetric assay kit (Biovision) according to manufacturer's instructions. Cells were lysed in buffer provided by the kit. GSH, total glutathione, and their ratio were determined by measuring reaction of reporter dye with GSH at 405 nm using a microplate reader.

Oxygen consumption

Basal oxygen consumption rate (OCR) was measured using a Clark-type O₂ electrode (Oxygraph+, Hansatech Instruments). Cells or tissue were placed in a chamber with 1 mL serum-free DMEM medium at 37°C and respiration was assessed over 1 minute. Control traces using medium alone were acquired after each sample to assess oxygen consumption attributed to the electrochemistry of the Clark electrode.

Seahorse analysis

OCR was measured using a Seahorse XFe24 Extracellular Flux Analyzer. Cells were seeded in Seahorse XFe24 microplates at ~60,000 cells/well and incubated overnight. Medium was then replaced with Seahorse XF Assay Medium, and cells were incubated in a CO₂-free incubator at 37°C for 1 hour prior to loading into the XFe24. The measurement protocol consisted of 3 minute mix, 2 minute wait, and 3 minute measurement cycles at 37°C, allowing for OCR measurements every 8 minutes. OCR measurements were normalized to protein concentration using the Bradford Assay. For the Mito Fuel Flex Kit, optimal concentrations of BPTES (3 µM), etomoxir (4 µM), and UK5099 (2 µM) were used.

HP ¹³C NMR spectroscopy of cell slurries

Approximately 24 µL of 14.2 M [1-¹³C]pyruvate with 15 mM trityl radical (GE Healthcare) was polarized in a HyperSense polarizer (Oxford Instruments) for 1 hr followed by rapid dissolution in a superheated isotonic buffer (4.5 mL) with 0.3 mM EDTA and equivalents of NaOH (80 mM) to yield a pH of 6.5-8. Each cell slurry of 10-40 million cells resuspended in 200 µL of serum-free DMEM medium was kept on ice and allowed to equilibrate to 37°C at the start of dissolution, then injected with equivalent volume of 80 mM HP [1-¹³C]pyruvate. Cell count did not affect results since 10 million cells were sufficient to fill the sensitive region of the coil. Dynamic ¹³C NMR spectra were acquired every 4s using 10° pulses at 37°C using a 60 MHz benchtop NMR spectrometer (Oxford Instruments) equipped with a 5-mm ¹H/¹³C probe. The spectra were zero-filled by a factor of 2, Fourier-transformed, phased, and baseline-corrected. Pyruvate and lactate peaks at each time point were manually fitted with a Lorentzian-Gaussian shape. The pyruvate-to-lactate conversion rate (k_{PL}) was characterized by fitting the dynamic pyruvate and lactate curves to a one-compartment model using nonlinear least squares fitting.

TRAMP Hyperpolarized ¹³C MRI

Hyperpolarized MR imaging of TRAMP mice was performed on a GE 3T human scanner with a custom-designed, dual-tuned mouse coil. However, to ensure adequate size of prostate tumors prior to hyperpolarized studies, mice were imaged on the 14T vertical bore Varian microimaging imaging system (Agilent Technologies). All studies were performed under the aegis of an animal protocol approved by the UCSF Institutional Animal Care and Use Committee. T₂-weighted ¹H anatomical MR images were acquired using a fast spin echo (FSE) sequence in sagittal, axial, and coronal views. Approximately 24 µL of 14.2 M [1-¹³C]pyruvate with 15 mM trityl radical (GE Healthcare) was polarized on a HyperSense polarizer (Oxford Instruments) for 1 hr as previously described [10] followed by dissolution in 4.5 mL 50 mM phosphate buffer with 0.3 mM EDTA and equivalents of NaOH (80 mM) to obtain physiological temperature and pH. Approximately 300 µL hyperpolarized solution was injected via a pre-established jugular or tail vein catheter over 12 sec followed by a chase of 150 µL of heparinized saline. A 3D CSI sequence was initiated at 35 sec after injection and HP-¹³C signal acquired

over 14 seconds. A double spin-echo pulse sequence with small tip-angle excitation, adiabatic refocusing and flyback echo-planar readout trajectory [10] and an 8x8x16 matrix and 40 x 40 x 86.4 mm field-of-view (0.135 cc resolution) was used to acquire *in vivo* 3D hyperpolarized ¹³C MRSI data in 14s after the animals were injected with hyperpolarized [1-¹³C] pyruvate. After baseline imaging the mice underwent orchiectomy and the hyperpolarized imaging was repeated again ~5 days (± 2 days) later. Mice were categorized into ADPC and CRPC as detailed above following the modified RECIST criteria.

Statistics

Student's *t*-test was used to compare human and murine ADPC and CRPC models using Prism (GraphPad) version 8.3. All statistics are reported as mean ± standard error. P values less than 0.05 (**p* < 0.05, ***p* < 0.01 and ****p* < 0.001) were considered significant.

5. Conclusions

In summary, our findings using human and murine models are consistent with the hypothesis that castration-resistant growth alters several metabolic pathways, including glycolysis, glutaminolysis, TCA metabolism, and glutathione redox capacity. These pathways may provide biomarkers to predict the development of clinical CRPC or candidate drug targets for the treatment of CRPC in patients.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1

Author Contributions: Conceptualization, J.S., R.S., J.K. R.A.; methodology, J.S., R.S., J.K.; validation, R.S., D.U.; Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.S., J.D.S., R.B., R.D.S., R.S., J.K.; formal analysis, J.S., R.S., J.D.S., D.U.; investigation, J.S., R.S., J.D.S., M.V.C.; resources, D.V., R.A., R.S., J.K., M.V.C.; data curation, R.S., D.U.; writing—original draft preparation, J.S., D.M.P., R.S. and J.K.; writing—review and editing, J.S., D.M.P., R.S. and J.K.; visualization, J.S., R.S.; supervision, R.S., J.K.; project administration, R.S., J.K.; funding acquisition, R.S., J.K., and D.B.V. All authors have read and agreed to the published version of the manuscript.

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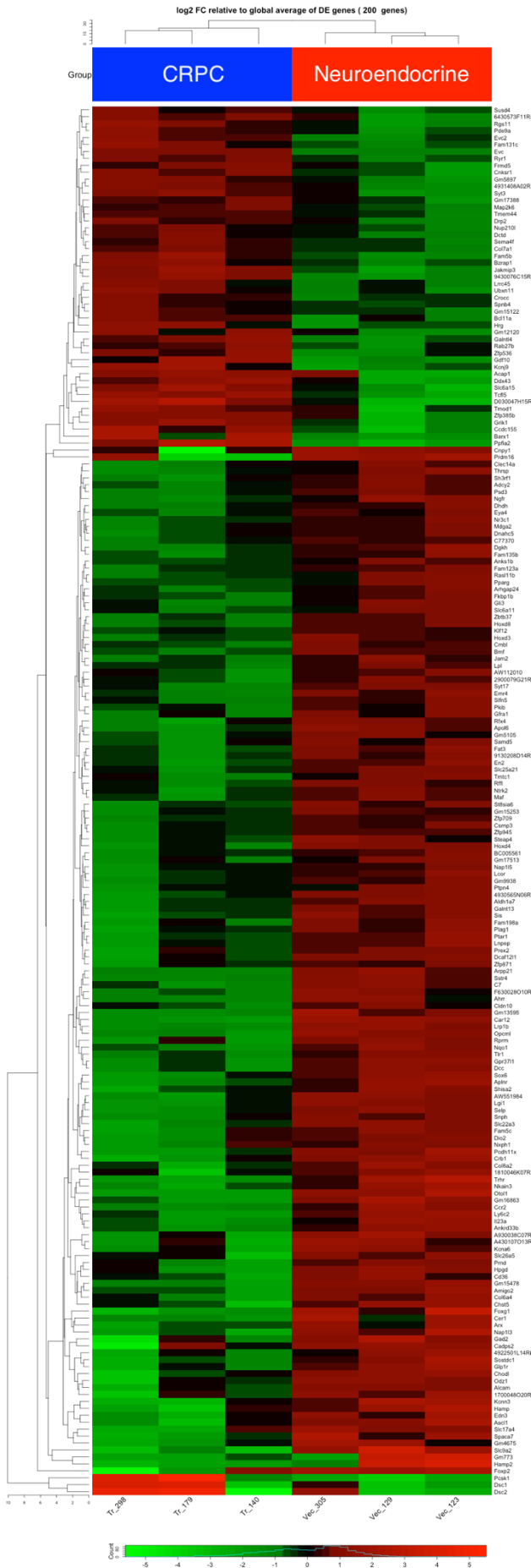
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Appendix 3
Heatmap of differential gene expression between CRPC and NEPC TRAMP tumors



Metabolic Adaptations of Treatment-Emergent Small Cell Neuroendocrine Prostate Cancer using Patient-Derived Xenografts

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Synopsis

This [U-¹³C]glucose and [U-¹³C]glutamine labeling study demonstrated that treatment-emergent small cell neuroendocrine prostate cancer PDX xenografts had significantly increased alanine synthesis (2-fold), glycolysis (1.5-fold), TCA metabolism (2.3-fold) and glutaminolysis (2-fold) compared to adenocarcinoma castrate resistant prostate cancer PDXs. This data suggests a combination of hyperpolarized [1-¹³C]pyruvate, [2-¹³C]pyruvate and [5-¹³C]glutamine could noninvasively distinguish these advanced prostate cancer phenotypes in future hyperpolarized ¹³C MRI patient exams for improved treatment selection and monitoring of advanced prostate cancer patients.

Introduction

Treatment-emergent small cell neuroendocrine prostate cancer (NEPC) is an increasingly prevalent, lethal subtype of castrate resistant prostate cancer (CRPC) that arises as an adaptive response to androgen deprivation therapy (ADT) and second-generation potent androgen pathway inhibitors (APIs) such as abiraterone, apalutamide and enzalutamide¹. Significant heterogeneity underlies the biology of CRPC, with frequent admixture of adenocarcinoma (aCRPC) and small cell (NEPC) histology within and across CRPC patients. Given the multiple therapeutic modalities currently available and being developed for NEPC, including platinum-based chemotherapy, inhibitors of glutamine metabolism (e.g. CB-839), and other MYC-directed treatment strategies (e.g. bromodomain inhibitors), there is a clear need to develop a non-invasive imaging modality to identify the presence of NEPC and provide real-time monitoring of metabolic response to treatment^{2,3}. The goal of this study was to identify metabolic differences between aCRPC and NEPC in order to determine the best combination of hyperpolarized ¹³C MRI probes that can discriminate between aCRPC and NEPC in future patient studies. Here, principal component analysis (PCA) and ¹³C-glucose and ¹³C-glutamine labeling methods were performed to characterize flux through glycolysis, tricarboxylic acid (TCA) cycle, alanine synthesis and glutaminolysis in aCRPC and NEPC patient-derived xenograft (PDX) models.

Methods

PDX models and ¹³C labeling: aCRPC (LTL-313HR, LuCaP 70CR, LuCaP 77CR) and NEPC (LTL-352, LTL-610, LuCaP 93, LuCaP 145.1) xenograft lines^{4,5} (**Fig1**) were transplanted into the subrenal capsule of male NSG mice. Mice with tumors were injected with [U-¹³C]glucose or [U-¹³C]glutamine via tail vein over 45min⁶. Tissue was flash-frozen immediately upon sacrifice.

Sample Preparation: Aqueous metabolites were extracted using cold methanol:water:chloroform⁷ and resuspended in D₂O.

NMR: High-resolution NMR spectra were acquired on an 800Mhz Bruker Avancel equipped with a multichannel cryo-probe (**Fig2**). ¹H presaturation with and without ¹³C-decoupling: TD=24k, SW=15ppm, TR=12s, NS=32. ¹H-¹³C HSQC: TD=2048x4096k, SW=6x120ppm, TR=1.5s, AQ=0.2s, NS=8, J_{CH}=135Hz.

PCA: Spectra were phased and baselined in MestreNova. Water region (4.6-4.9ppm) was removed, and remaining spectra (0.7-5.8ppm) were divided into 0.03ppm bins. PCA with probabilistic quotient normalization and pareto scaling was performed using ChemoSpec⁸.

Metabolite quantification: Fractional enrichment (FE) was quantified as FE=[¹³C-metabolite]/[total metabolite]. Total metabolite concentrations were quantified from ¹³C-decoupled ¹H spectra with the same preprocessing as PCA. ¹³C-labeled metabolite concentrations were quantified from 2D ¹H-¹³C HSQC. A subset of LTL313HR PDXs labeled with [U-¹³C]glucose is currently being processed for quantification of steady-state metabolite quantification and fractional enrichment. Statistical significance was determined using student's t-test. All data are shown as mean±standard error.

Results and Discussion

Representative ¹³C-decoupled ¹H spectra from aCRPC and NEPC tumor extracts are shown in **Fig2A**. Based on known genetic modification underlying NEPC transdifferentiation, there are multiple metabolic dysregulations that are implicated in this disease⁹. However, variations in intracellular metabolite concentrations between tumor phenotypes were relatively small due to metabolic homeostasis. Quantification of steady state metabolite pools did not show a significant difference in any metabolite other than lactate between the two phenotypes (**Fig2B**). NEPC tumors had a significantly increased lactate concentration (80±6 vs 44±6.5, p<0.01) compared to aCRPC. This necessitates the use of ¹³C-labeled tracers to uncover a clear picture of the key pathways implicated in the pathophysiology of NEPC transdifferentiation.

Consequently, the ¹³C isotopomer flux pattern imposes an extremely complex, overcrowded, spectrum with overlapping peaks from different metabolites. This makes manual interpretation of 1D NMR spectra difficult. However chemometric techniques such as non-targeted PCA can provide unique insights. Here, aCRPC and NEPC groups were differentiated with 95% confidence using PCA analysis of the ¹H spectra, which reflect changes associated with ¹³C-labeling of both glucose and glutamine (**Fig3A and 3D**). PCA loadings plots (**Fig3B and 3E**) show the influence of each spectral bin. The most influential spectral bins were identified using an s-shaped plot. For [U-¹³C]glucose-labeled extracts, spectral bins corresponding to lactate, alanine, glucose, metabolites in the 3.2-4.2ppm range, glutamate and ¹³C-lactate satellite peaks had the greatest influence in differentiating the groups (**Fig3C**). For [U-¹³C]glutamine-labeled extracts, spectral bins corresponding to glutamate, glutamine, and metabolites in the 3.2-4.2ppm range had the most contribution (**Fig3F**). To corroborate this finding, the fractional enrichment of pertinent metabolites was quantified using 1D ¹³C-decoupled ¹H and 2D HSQC NMR spectroscopy (**Fig4A**). Following [U-¹³C]glucose infusion, NEPC tumors had elevated alanine FE (44±2 vs 22±3, p<0.001), glutamate FE (23±2 vs 10±3, p<0.01), and lactate FE (46±2 vs 31±2, p<0.01) compared to aCRPC tumors (**Fig4B**). With [U-¹³C]glutamine infusion, NEPC tumors also had elevated aspartate FE (19±2 vs 10±2, p<0.01) and glutamate FE (28±3 vs 14±2, p<0.01) compared to aCRPC tumors (**Fig4C**). Together these results suggest that alanine synthesis, glycolysis, TCA metabolism, and glutaminolysis are upregulated in NEPC (**Fig5**). This is consistent with our hypothesis that these pathways may be upregulated in NEPC due to MYC overexpression⁹.

Conclusion

This study demonstrated that NEPC PDXs had significantly increased glucose and glutamine metabolism compared to aCRPC PDXs. We also showed that PCA of 1D proton spectra can be used to differentiate between the two phenotypes, and is reflective of the isotopomer flux pattern. Future work will correlate these findings with whole transcriptome analysis to understand the underlying basis of the metabolic alterations observed in this study. Based on these metabolic differences, we propose using a combination of hyperpolarized [1-¹³C]pyruvate, [2-¹³C]pyruvate and [5-¹³C]glutamine to noninvasively assess alanine synthesis and glycolysis, TCA metabolism, and glutaminolysis respectively in future patient studies using hyperpolarized ¹³C MRI.

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Figures

PDX	Phenotype	Site of Origin	N _{glucose}	N _{glutamine}
LTL-313HR	CRPC	Primary PCa	3	4
LuCaP 70CR	CRPC	Liver Metastasis	1	0
LuCaP 77CR	CRPC	Femur Metastasis	3	3
LTL-352	NEPC	Urethral Metastasis	3	3
LTL-610	NEPC	Primary NEPC	3	3
LuCaP 93	NEPC	Primary NEPC	3	3
LuCaP 145.1	NEPC	Liver Metastasis	3	3

Figure 1. List of PDXs used in this study with model with tumor histology, site of origin, and sample size of mice infused with [U-¹³C]glucose and [U-¹³C]glutamine.

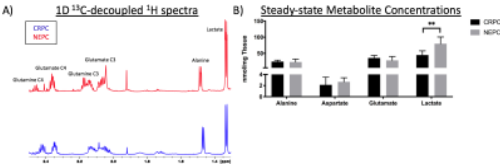


Figure 2. Steady state metabolic profiling using 1D proton NMR. (A) Representative high-resolution ¹³C-decoupled ¹H NMR spectra of aCRPC and NEPC extracts (B) Steady-state metabolite concentrations of aCRPC (n=4) and NEPC (n=12) extracts were quantified from 1D ¹³C-decoupled ¹H spectra. Data shown is mean ± standard error. **p<0.01

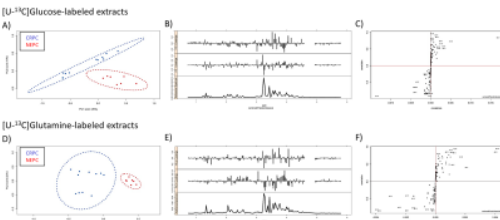


Figure 3. Principal Component Analysis was performed using ^1H spectra of aCRPC (n=7) and NEPC (n=12) extracts labeled with $[\text{U-}^{13}\text{C}]$ glucose (top row: A,B,C) and $[\text{U-}^{13}\text{C}]$ glutamine (bottom row: D,E,F). (A,D) PCA score plots, (B,E) PCA loadings score plots, and (C,F) PCA loadings s-shaped plots. Dotted ellipses indicate 95% confidence interval for classification.

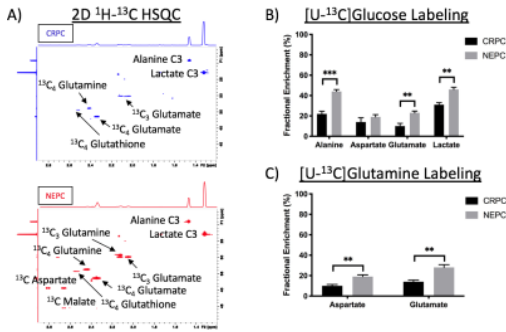


Figure 4. Comparison of fractional enrichments of key metabolites arising from ^{13}C -labeling of aCRPC and NEPC PDXs. (A) Representative high-resolution 2D ^1H - ^{13}C HSQC of aCRPC and NEPC extracts. (B) After $[\text{U-}^{13}\text{C}]$ glucose infusion, NEPC (n=12) tumors had significantly elevated alanine FE, glutamate FE, and lactate FE compared to aCRPC (n=4) tumors. (C) After $[\text{U-}^{13}\text{C}]$ glutamine infusion, NEPC (n=12) tumors had significantly elevated aspartate FE and glutamate FE compared to aCRPC (n=7) tumors. Data is presented as mean $\pm$ standard error. **p<0.01, ***p<0.001.

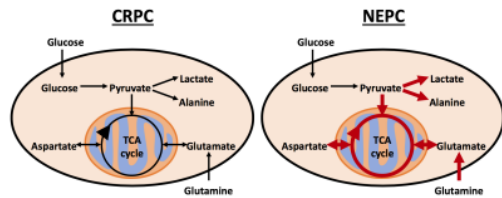


Figure 5. Schematic detailing the changes in glucose and glutamine metabolism observed between aCRPC and NEPC tumors.

NMR quantification of lactate production and efflux and glutamate fractional enrichment in living human prostate biopsies cultured with [1,6-¹³C₂]glucose

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Purpose: Image-guided prostate biopsies are routinely acquired in the diagnosis and treatment monitoring of prostate cancer, yielding useful tissue for identifying metabolic biomarkers and therapeutic targets. We developed an optimized biopsy tissue culture protocol in combination with [1,6-¹³C₂]glucose labeling and quantitative high-resolution NMR to measure glycolysis and tricarboxylic acid (TCA) cycle activity in freshly acquired living human prostate biopsies.

Methods: We acquired 34 MRI-ultrasound fusion-guided prostate biopsies in vials on ice from 22 previously untreated patients. Within 15 min, biopsies were transferred to rotary tissue culture in 37°C prostate medium containing [1,6-¹³C₂]glucose. Following 24 h of culture, tissue lactate and glutamate pool sizes and fractional enrichments were quantified using quantitative ¹H high resolution magic angle spinning Carr-Purcell-Meiboom-Gill (CPMG) spectroscopy at 1°C with and without ¹³C decoupling. Lactate effluxed from the biopsy tissue was quantified in the culture medium using quantitative solution-state high-resolution NMR.

Results: Lactate concentration in low-grade cancer (1.15 ± 0.78 nmol/mg) and benign (0.74 ± 0.15 nmol/mg) biopsies agreed with prior published measurements of snap-frozen biopsies. There was substantial fractional enrichment of [3-¹³C]lactate (≈70%) and [4-¹³C]glutamate (≈24%) in both low-grade cancer and benign biopsies. Although a significant difference in tissue [3-¹³C]lactate fractional enrichment was not observed, lactate efflux was significantly higher (*P* < 0.05) in low-grade cancer biopsies (0.55 ± 0.14 nmol/min/mg) versus benign biopsies (0.31 ± 0.04 nmol/min/mg).

Conclusion: A protocol was developed for quantification of lactate production-efflux and TCA cycle activity in single living human prostate biopsies, allowing metabolic labeling on a wide spectrum of human tissues (e.g., metastatic, post-non-surgical therapy) from patients not receiving surgery.

KEYWORDS

biopsy, efflux, lactate, metabolism, prostate, tracer

1 | INTRODUCTION

Prostate cancer metabolism is characterized by a shift to aerobic glycolysis and lactate production, known as the Warburg effect.¹⁻⁵ Yet, it is not currently known how tumor metabolism changes in the setting of human castration-resistant and/or neuroendocrine prostate cancer (CRPC/NEPC). Recent studies have identified genetic alterations with significant putative metabolic effects in human CRPC.^{6,7} To investigate these effects, it is essential to measure metabolism in living human prostate tissue. Our laboratory has previously used an optimized rotary tissue culture system to perform ¹³C NMR metabolic labeling studies in slices of human prostate tissue acquired from radical prostatectomy (RP).⁸⁻¹⁰ Yet, RP is not standard of care for CRPC or NEPC, so the tissue slice technique cannot be applied in this clinically significant cohort of patients to yield a better insight into their respective metabolic adaptations. Although it is clinically feasible to obtain biopsy samples from patients with CRPC and/or NEPC, a novel metabolic profiling protocol that is optimized for living human biopsies is needed to study these specimens.

In the current work, we demonstrate that the combination of rotary biopsy culture, ¹³C metabolic labeling, and NMR can quantify metabolism in freshly acquired living human prostate biopsies. The current research focuses on the development of the techniques necessary to acquire and measure the Warburg effect in living patient-derived biopsies, acquired before treatment for the determination of cancer presence, Gleason grade, and extent for personalized therapeutic selection. By definition, this was an early stage cancer patient population, resulting in the acquisition of mainly benign and low grade (primary Gleason pattern 3) prostate cancer biopsies in this study. However, the ultimate goal is to use the optimized techniques developed in this study to assess the Warburg effect and other changes in metabolism in biopsies from patients with advanced disease who would not normally receive surgical therapy.

A particular motivation for studying the metabolism of prostate cancer comes from the preclinical and clinical development of hyperpolarized ¹³C MRI techniques,¹¹⁻¹⁴ with clinical trials of hyperpolarized [1-¹³C]pyruvate in prostate cancer now underway at multiple sites.¹⁵ In prostate cancer, there is considerable heterogeneity of prognosis for patients, particularly for those with a cancer of Gleason 7 histopathological score or lower.¹⁶ Therefore, there is significant potential for hyperpolarized ¹³C metabolic imaging of primary prostate cancer to provide additional information to the clinician, in combination with Gleason scoring and clinical risk

scores such as the Cancer of the Prostate Risk Assessment (CAPRA) score.¹⁷ In the metastatic setting, it is widely recognized that metabolic responses to therapy can significantly precede anatomic responses,¹⁸ which means that hyperpolarized ¹³C metabolic imaging may be able to better inform in real-time spatially resolved treatment monitoring for the clinician and patient in conjunction with serum tumor markers such as prostate-specific antigen (PSA).¹⁹

In hyperpolarized ¹³C MRI, the measured signal is proportional to the product of (1) probe polarization, (2) the fractional enrichment of the precursor being converted to the produced metabolite, and (3) the pool size of the produced metabolite.²⁰ In the ex vivo setting, it becomes possible to disentangle these factors, ideally leading to a better interpretation and understanding of the hyperpolarized ¹³C signal¹⁰ and enabling the development of new hyperpolarized probes and potential therapeutic targets.

A significant challenge in the metabolic profiling of specific biochemical pathways using stable isotopes in individual living prostate biopsies by NMR is the small tissue mass ranging from 1–6 mg, which limits the achievable SNR. Therefore, in the present work, we have focused on optimizing the experimental parameters such as the duration of time in rotary biopsy culture and the parameters of the NMR acquisition so as to achieve sufficient metabolite signals from a small mass of tissue incubated with ¹³C-labeled metabolic substrates.

2 | METHODS

2.1 | Biopsy acquisition

Transrectal 18-gauge prostate biopsies from 22 previously untreated patients (34 total biopsies, 27 normal prostate biopsies, 5 Gleason 6(3+3) cancer biopsies, and 2 Gleason 7(3+4) cancer biopsies) were acquired with Institutional Review Board approval. Biopsies were acquired using an ultrasound-MRI fusion system that provided registration of transrectal ultrasound images to prior prostate MRI (UroNav, InVivo, Gainesville, FL). The clinical efficacy of this system has been evaluated previously.²¹ Up to 2 research biopsies per patient were acquired from any visible multiparametric ¹H MRI or ultrasound lesions once the clinical biopsy acquisition had been completed. In cases with fewer than 2 visible lesions, a biopsy was sometimes taken from a normal-appearing area of the peripheral zone. Immediately following acquisition, biopsies were stored in vials on ice and were transferred to rotary culture within 15 min.

2.2 | Biopsy culture medium

A formulation of PFMR-4A medium with supplements optimized for prostate tissue culture was previously described.^{8,9} For this study, it was found that HEPES and threonine in this medium posed a potential challenge for the quantification of NMR spectra. Consequently, we developed a formulation of bicarbonate-buffered, HEPES-free, threonine-free, pyruvate-free F-12 medium containing all of the previously described supplements.^{8,9} (The complete medium recipe is provided in Supporting Information Tables S1 and S2). For labeling studies, the medium contained exclusively [1,6-¹³C₂]glucose at 1.0 g/L.

2.3 | Biopsy rotary tissue culture

Techniques previously described for the culture of thin, precision-cut prostate tissue slices⁸ were adapted for culture of biopsies. Each biopsy was loaded onto a titanium mesh inside of 1 well of a 6-well plate containing 2.5 mL per well

of pre-warmed F-12 prostate medium with [1,6-¹³C₂]glucose. The plates were loaded onto a rotary tissue culture system (Alabama R&D) (Figure 1B) within a 37°C, 5% CO₂ incubator, and the biopsies were cultured for either 24 or 26 h. For a subset of samples, the biopsy culture medium was collected and replaced with fresh pre-warmed medium at the 2-h time point to quantify the initial rate of lactate efflux, in addition to the lactate efflux after 24 h of culture. Each 6-well plate also contained a control well containing medium but without any biopsy tissue. At the end of the culture period, the medium was collected and stored at -80°C.

2.4 | Biopsy rinsing

After culture, the biopsies were placed in ice-cold phosphate-buffered saline for <1 min, blotted with a delicate task wipe, and stored at -80°C. This procedure was intended to remove medium that could otherwise contaminate the

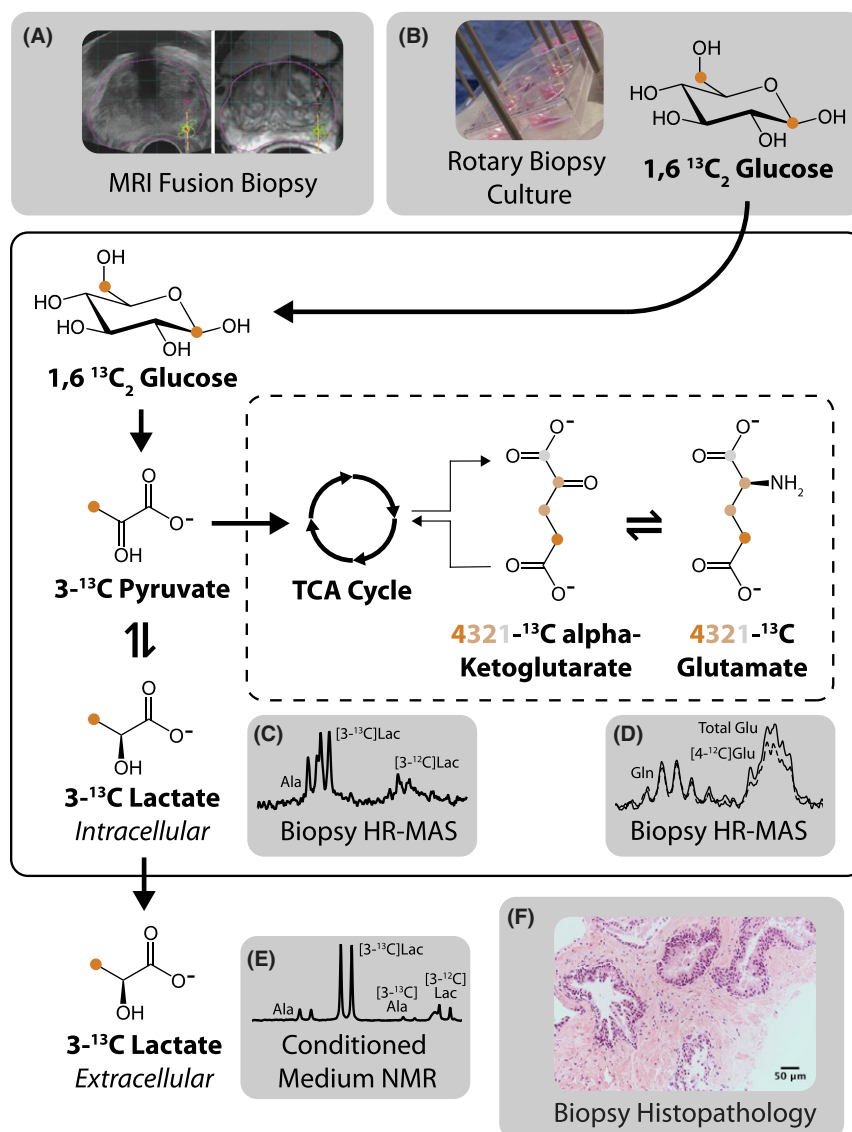


FIGURE 1 Study protocol. (A) MRI fusion biopsy was followed by (B) rotary tissue culture with [1,6-¹³C₂]glucose. (C) Tissue lactate and (D) glutamate concentration and fractional enrichment were quantified using HR-MAS of biopsy samples, whereas (E) lactate efflux in media was quantified using NMR. (F) The biopsy histopathology was obtained after culture

post-culture biopsy high resolution-magic angle spinning (HR-MAS) NMR spectra as we have shown previously.²²

2.5 | Biopsy tissue NMR measurements

Frozen biopsies were loaded without thawing into a sealed zirconium HR-MAS rotor^{22,23} on a tared digital balance to measure the wet weight of the biopsy tissue. Six μL of $\text{D}_2\text{O}/0.05\%$ trimethylsilylpropanoic acid (TSP) solution was further added to the rotor and weighed to enable deuterium locking as well as a chemical shift reference via TSP. The HR-MAS rotor was sealed and loaded into a 500 MHz Varian gHX Nano (proton-carbon double resonance) 4 mm indirect detection HR-MAS probe maintained at 1°C . Acquisition was performed at a spin rate of 2.25 kHz using a Varian Inova console with VNMRJ 4 after locking on the D_2O signal. The probe tuning, 90° pulse width and water saturation frequency were calibrated for each sample. Each sample was shimmed using an automated FID shimming routine for 20 min (see Supplemental Information). ^1H Carr-Purcell-Meiboom-Gill (CPMG) spectra were acquired with 60,000 complex points, 3-s acquisition time, 2-s water suppression pulse, 6-s overall repetition time, and 1024 scans. It was previously established that this repetition time yielded fully relaxed spectra in human prostate tissue.²⁴ The CPMG TE was set to 288 ms. The spacings of 180° pulses within the CPMG sequence were synchronized to the spinning period of the rotor.²⁴ To quantify metabolite total pools in addition to ^{13}C -labeled fractions, CPMG data sets were acquired both with and without heteronuclear ^{13}C decoupling using a GARP pulse^{22,25} for a total CPMG scan time of 3 h, 25 min.

2.6 | Biopsy histopathology

Following the HR-MAS data acquisition, the biopsies were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and high-molecular weight cytokeratin for interpretation by a board-certified genitourinary pathologist. For each sample the pathologist determined the primary Gleason pattern, secondary Gleason pattern, Gleason score, percentage of cancer, percentage of glandular tissue, and percentage of stromal tissue.

2.7 | Biopsy media NMR measurements

The samples of biopsy medium were thawed from storage at -80°C . NMR samples were prepared by adding 65 mg of $\text{D}_2\text{O}/0.05\%$ TSP to 540 mg of conditioned medium and placed in 5 mm NMR tubes. The sample data were acquired at 600 MHz using a Bruker Avance III console equipped with a 5 mm Bruker BBFO (broadband double resonance) direct detection probe. The 90° pulse was calibrated, and automated z-axis shimming was performed up to 8th order in addition

to tuning of all first-order shims (see Supporting Information Tables S3 and S4). Water-suppressed data were acquired using a fully relaxed ZGCPPR pulse sequence with a 12-s acquisition time (114,942 points) and 3-s relaxation delay and 32 scans (8 min per sample).

2.8 | NMR data processing

All NMR data sets were processed in MestreNova 12 (Mestrelab Research S.L.). The data sets were zero-padded by a factor of 2, apodized with a 0.25 Hz exponential filter, and automatically phased and baselined using a Whittaker smoother algorithm²⁶ and manually adjusted as needed. Peaks of interest were automatically picked and fitted using a Lorentzian-Gaussian shape function with a simulated annealing algorithm (500 coarse iterations, 100 fine iterations, and a local minima filter of 25 were used). All automated peak fits were tweaked after visual assessment for fit quality based on the residual signal and adjusted when necessary.

2.9 | NMR quantification

In the biopsy HR-MAS CPMG data, peak areas were quantified relative to the calibrated amplitude of the electronic reference to access in vivo concentrations (ERETIC) signal, corrected for the number of metabolite protons.²³ Appropriate T_2 corrections were applied based on HR-MAS measurements in human prostate tissue at 1°C and 500 MHz (lactate $\text{C3 } T_2 = 251$ ms, glutamate $\text{C4 } T_2 = 275$ ms).^{4,10} Metabolite amounts were further standardized by the wet tissue weight of the sample, measured after culture, to yield a biopsy metabolite concentration in (nmol/mg). Fractional enrichments were computed as follows.

The glutamate fractional enrichment at the 4-position was computed by differencing the quantification of the 2.34 ppm C4 multiplet in the ^{13}C decoupled versus non-decoupled CPMG spectrum. This obviated the need to quantify the $[4-^{13}\text{C}]$ glutamate satellite peaks, which are known to underlie other peaks such as the $[4-^{12}\text{C}]$ glutamine peak and additionally could be further split by the presence of $[3-^{13}\text{C}]$ glutamate labeling on the same molecule (Figure 1D).

In the fully relaxed media data, the lactate peak area was quantified relative to the known peak area and measured mass of TSP and corrected for the relative number of protons. The lactate amount was further standardized by the wet tissue weight of the biopsy, as well as the duration of the biopsy tissue culture, to yield a lactate efflux rate in (nmol/min)/(mg tissue).

2.10 | LIVE/DEAD assays and imaging

At the end of the culture experiment, a limited number of biopsies were transferred to a new well containing medium

with ethidium homodimer-1 at 0.5 $\mu\text{mol/L}$ and calcein-AM at 2.5 $\mu\text{mol/L}$.²⁷ The biopsies were maintained in culture for a further 2 h and then rinsed for 10 min in phosphate-buffered saline under dark conditions before blotting with a delicate task wipe and imaging on a laser scanning confocal microscope (Zeiss 780). A two-photon excitation was used at a 759 nm wavelength with a $25\times$ water immersion objective, a 1024×1024 image size, and 2 averages. The detection windows were set as 493–556 nm for green and 615–741 nm for red. Z-stack images were acquired using overlapping optical sectioning with thickness determined by 1 Airy unit, with the maximum possible Z-stack height to assess tissue viability throughout the sample. We found that our extended staining protocol allowed the stain to diffuse throughout the biopsy, which made it possible to acquire a Z-stack covering the entire biopsy thickness ($\sim 400\ \mu\text{m}$). Because of the time spent at room temperature on the microscope, the samples analyzed in this manner were not used for any HR-MAS data acquisition after imaging.

3 | RESULTS

3.1 | Overview of study protocol

The MRI fusion biopsy procedure provided registration of ultrasound to multiparametric prostate MRI, with the MRI lesion highlighted for biopsy targeting (Figure 1A). The freshly acquired biopsies were cultured with $[1,6\text{-}^{13}\text{C}_2]$ glucose that was converted into $[3\text{-}^{13}\text{C}]$ pyruvate via glycolysis (Figure 1B). The pyruvate interconverted with the intracellular lactate pool, which was quantified after culture using HR-MAS. In Figure 1C, the 500 MHz HR-MAS spectrum shows doublets of $[3\text{-}^{12}\text{C}]$ alanine, $[3\text{-}^{13}\text{C}]$ lactate, and $[3\text{-}^{12}\text{C}]$ lactate going from left to right (total scan time for this spectrum: 1 h, 42 min). The pyruvate also entered the TCA cycle via pyruvate dehydrogenase, which led to labeling at the 4-position of α -ketoglutarate following 1 turn of the cycle. This interconverted with glutamate to produce labeling at the 4-position of glutamate. Figure 1D shows the higher amplitude of the multiplets corresponding to the C4 protons of glutamate when decoupling is turned on (total scan time for this spectrum, with decoupling both off and on: 3 h, 25 min). Figure 1E shows a representative spectrum of the medium signifying the efflux of lactate and alanine from the biopsy tissue as evidenced by the ^{13}C satellite peaks of the respective methyl protons. The biopsy histopathology after culture demonstrated well-defined glands with preserved tissue microarchitecture (Figure 1F: H&E staining, $20\times$ magnification). The representative biopsy was benign with 30% glandular tissue and 70% stromal tissue (a detail of a glandular region is shown).

3.2 | Study data set

We obtained a total of 34 biopsies from 22 previously untreated patients. Of these biopsies, 16 were targeted to a radiologist-identified MRI lesion using the MRI-ultrasound fusion biopsy device. Twenty-seven benign biopsies and 7 cancer biopsies were acquired. The mean $\pm$ SE wet tissue weights were 3.65 ± 0.28 mg for benign biopsies and 4.40 ± 0.49 mg for cancer biopsies. Among the benign biopsies, the average percentage of benign glandular tissue was 17% as determined by a board-certified genitourinary pathologist. Of the 7 cancer biopsies, 5 had a Gleason score of 6(3 + 3) and 2 had a Gleason score of 7(3 + 4). The average percentage of cancer within the cancer biopsies was 12%, and the average percentage of benign glandular tissue was 9%. Of the 7 cancer biopsies, 4 were acquired from MRI-fusion targeted regions and 3 were acquired from non-MRI targeted regions.

3.3 | Biopsy media NMR measurements

We observed considerable efflux of $[3\text{-}^{13}\text{C}]$ lactate into the medium during culture. In the representative NMR spectra normalized to tissue mass shown in Figure 2A, the cancer (yellow) medium after 24 h of culture demonstrated a higher level of $[3\text{-}^{13}\text{C}]$ lactate efflux compared to the benign (blue) medium.

As shown in Figure 2B, benign biopsies had a $[3\text{-}^{13}\text{C}]$ lactate efflux rate of 0.29 ± 0.04 nmol/min/mg (mean $\pm$ SE), whereas cancer biopsies had a $[3\text{-}^{13}\text{C}]$ lactate efflux rate of 0.52 ± 0.14 nmol/min/mg measured over 24 h in culture. Benign biopsies had a total lactate efflux rate of 0.31 ± 0.04 nmol/min/mg (mean $\pm$ SE), whereas cancer biopsies had a total lactate efflux rate of 0.55 ± 0.14 nmol/min/mg measured over 24 h in culture. Both the $3\text{-}^{13}\text{C}$ and total lactate efflux rates were significantly higher for the cancer biopsies ($P < 0.05$, two-tailed Student's t-test for independent samples). The fractional enrichment of the effluxed lactate was $95 \pm 1\%$ for benign biopsies and $94 \pm 1\%$ for cancer biopsies, with no significant fractional enrichment differences observed between benign and cancer biopsies.

In an effort to assess the stability of lactate efflux over time in culture, for the majority of biopsies the lactate efflux rate per minute was measured from media collected after 2 h of biopsy culture ("0–2 h") and again from media collected after a further 24 h of biopsy culture ("2–26 h"). We did not observe significant differences between the lactate efflux rates measured over these 2 time points (Figure 3A: benign 0–2 h: 0.33 ± 0.05 nmol/min/mg, benign 2–26 h: 0.28 ± 0.03 nmol/min/mg; and 3B: cancer 0–2 h: 0.49 ± 0.10 nmol/min/mg, cancer 2–26 h: 0.53 ± 0.23 nmol/min/mg).

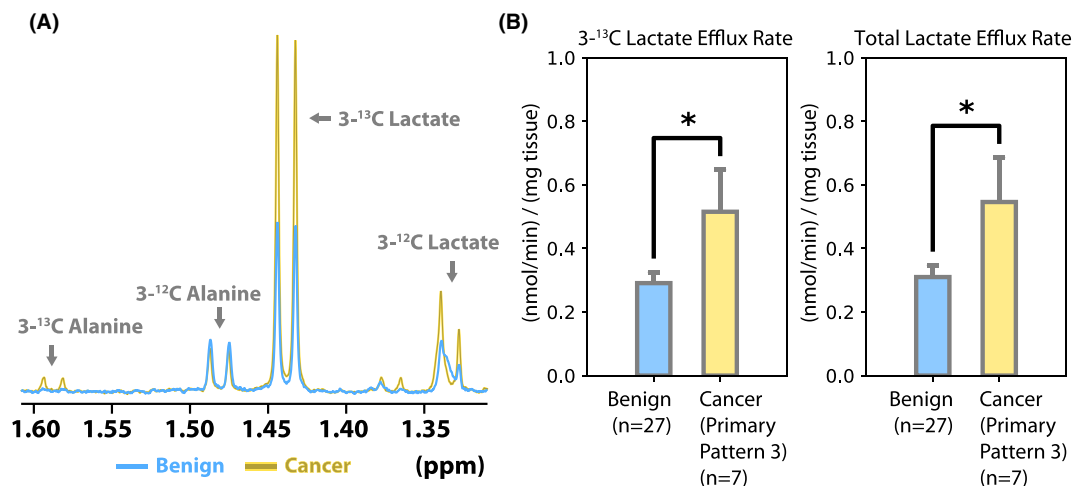


FIGURE 2 Biopsy lactate efflux and concentration. (A) Representative NMR spectra from benign (blue) and cancer (yellow) media. The broad peak that is superimposed with the 3-¹²C lactate doublet at 1.33 ppm is a medium additive that was deconvolved during the quantification process. (B) Quantification of [3-¹³C]lactate efflux and total lactate efflux in media. There was significantly greater [3-¹³C]lactate and total lactate efflux in cancer biopsies compared to benign biopsies ($P < 0.05$)

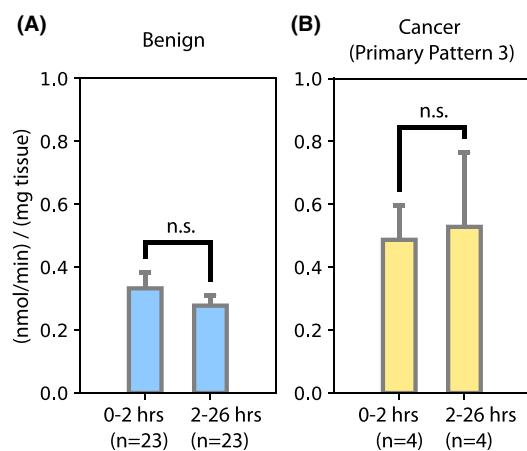


FIGURE 3 Biopsy lactate efflux over time. The biopsy lactate efflux rate per minute was measured from media collected after 2 h in culture ("0-2 h") and again collected after a further 24 h in culture with the same biopsy ("2-26 h")

3.4 | Biopsy tissue NMR measurements

Of the 34 biopsies cultured during this study, 7 were used for non-HR-MAS experiments after culture and 6 were not measured by HR-MAS because of technical issues. We obtained high-quality HR-MAS data from 21 biopsies suitable for metabolite quantification. Of these HR-MAS samples, 16 were benign, 4 were Gleason score 6(3 + 3), and 1 was Gleason score 7(3 + 4). It was possible to observe a number of metabolites in the HR-MAS CPMG spectra from biopsy tissues after culture, including metabolites that we have previously quantified in prostate tissue such as phospholipids²⁸ (Figure 4A). However, the focus of the present study was lactate production and efflux in culture. Despite a trend

toward higher lactate levels in the cancer biopsies, we did not observe significant differences between benign and cancer biopsies after culture (Figure 4B). The [3-¹³C]lactate concentration after culture was 0.56 ± 0.12 nmol/mg (mean $\pm$ SE) in benign biopsies and 0.73 ± 0.49 nmol/mg in cancer biopsies. The total lactate concentration after culture was 0.74 ± 0.15 nmol/mg (mean $\pm$ SE) in benign biopsies and 1.15 ± 0.78 nmol/mg in cancer biopsies. The fractional enrichment was $72 \pm 6\%$ in benign biopsies and $71 \pm 6\%$ in cancer biopsies.

3.5 | Labeling of glutamate from [1,6-¹³C₂] glucose

We were able to detect an increase of the glutamate C4 multiplet amplitude at 2.34 ppm with ¹³C decoupling turned on, indicating fractional enrichment (representative spectrum, Figure 5A). However, no significant differences were observed between the benign and cancer (primary pattern 3) biopsies in terms of the glutamate concentration and/or fractional enrichment (Figure 5B). As shown in Figure 5B, the [4-¹³C]glutamate concentration after culture was 0.32 ± 0.10 nmol/mg (mean $\pm$ SE) in benign biopsies and 0.27 ± 0.15 nmol/mg in cancer biopsies. The total glutamate concentration after culture was 1.18 ± 0.23 nmol/mg in benign biopsies and 0.92 ± 0.47 nmol/mg in cancer biopsies. The fractional enrichment of C4 glutamate after culture was $24 \pm 3\%$ in benign biopsies and $24 \pm 7\%$ in cancer biopsies.

3.6 | Biopsy LIVE/DEAD staining

We assessed the biopsy viability after 26 h in rotary culture using a fluorescent vital stain that is trapped in living cells (Calcein-AM) combined with a nuclear stain that enters

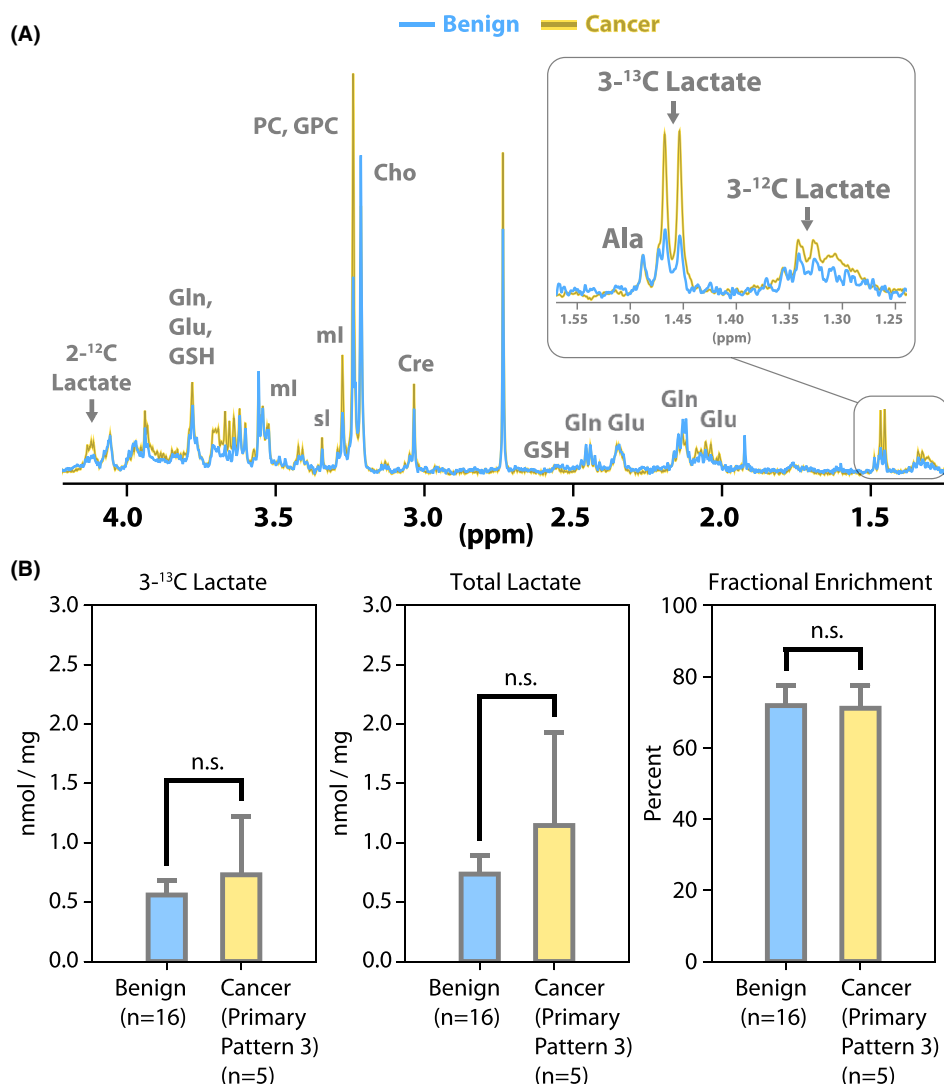


FIGURE 4 Biopsy tissue HR-MAS spectra. (A) Examples of CPMG spectra from benign (blue) and cancer (yellow) samples after culture. The [2-¹²C]lactate peak is visible at left, as a quartet split by the long-range coupling (~4 Hz) to [3-¹³C]lactate. Ala, alanine; Glu, glutamate; Gln, glutamine; GSH, glutathione; Cre, creatine; Cho, choline; PC, phosphocholine; GPC, glycerophosphocholine; mI, myo-inositol; sI, scyllo-inositol. (B) Quantified CPMG data: [3-¹³C]lactate concentration, total lactate concentration and fractional enrichment of lactate at the 3-position

the nuclei of dead cells (ethidium homodimer-1). Scattered cells demonstrated staining with ethidium homodimer-1, but the biopsies were primarily stained by the calcein-AM (Figure 6A), indicating that the majority of cells remained viable in biopsies after culture. The corresponding histopathology slides further demonstrated intact glandular architecture after culture (Figure 6B).

4 | DISCUSSION

In this study, we found that it was possible to perform NMR measurements of metabolism in individual living human prostate biopsies. Furthermore, the ¹³C-labeled and overall rates of lactate efflux in culture were significantly higher for low-grade (primary Gleason pattern 3) cancer biopsies as

compared to benign biopsies (Figure 2). In the HR-MAS data (Figure 4), for benign biopsies, we observed a tissue lactate concentration of 0.74 ± 0.15 nmol/mg, in good agreement with a prior published measurement of 0.61 ± 0.28 nmol/mg in snap-frozen biopsy samples by Tessem et al.⁴ For cancer biopsies, we observed a tissue lactate concentration of 1.15 ± 0.78 nmol/mg, as compared to a prior measurement of 1.59 ± 0.61 nmol/mg in snap-frozen cancer biopsies.⁴ This discrepancy can perhaps be explained by the fact that the present data set contained only low-grade prostate cancer biopsies (primary Gleason pattern 3), whereas the data set from Tessem et al.⁴ contained specimens of low- and high-grade prostate cancer (primary Gleason patterns 3 and 4). These findings are consistent with the notion that there is a more pronounced Warburg effect in high-grade prostate cancer such as primary Gleason pattern 4.²⁹

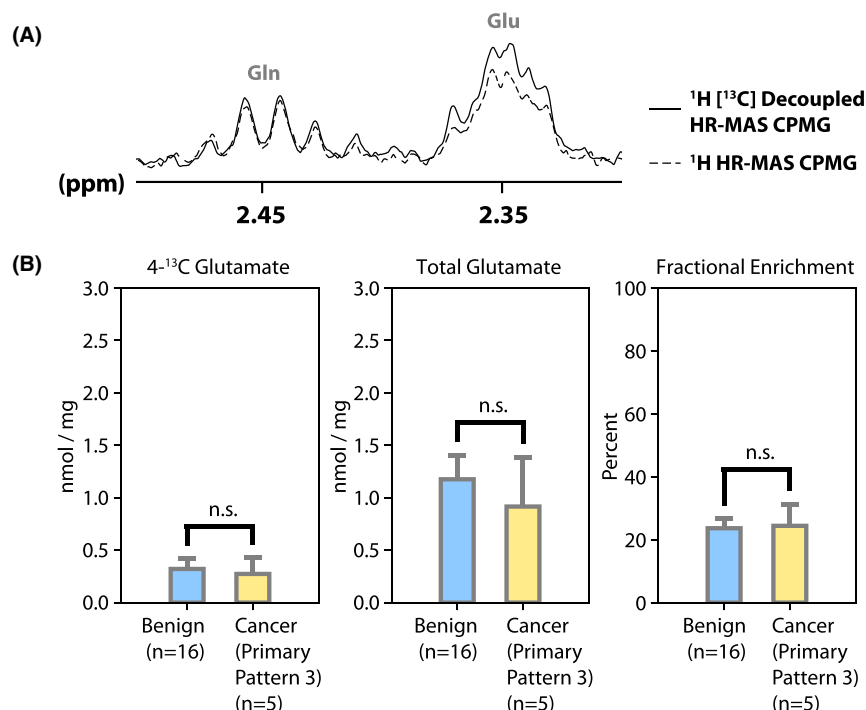


FIGURE 5 Labeling of glutamate from glucose. (A) There was an increase of the glutamate C4 multiplet amplitude at 2.34 ppm with ^{13}C decoupling turned on, indicating fractional enrichment. (B) Quantified CPMG data: 4- ^{13}C glutamate concentration, total glutamate concentration, and fractional enrichment of glutamate at the 4-position

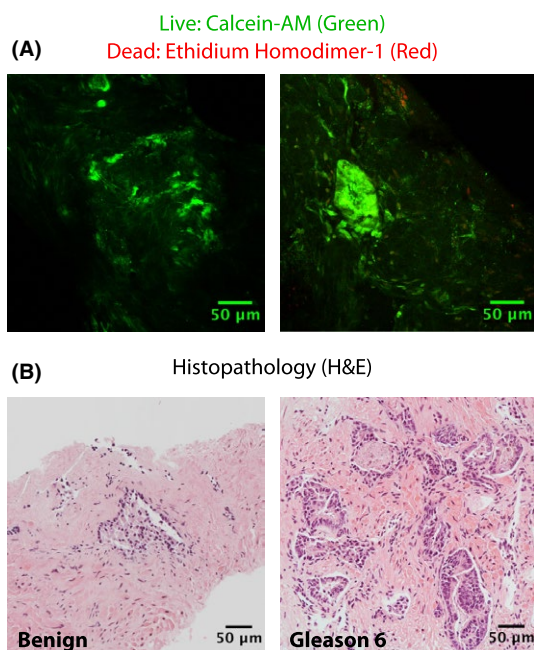


FIGURE 6 Biopsy LIVE/DEAD staining. (A) Confocal 2-photon microscopy from biopsy samples stained with calcein-AM and ethidium homodimer-1 following 26 h in culture. (B) Biopsy histopathology obtained after confocal microscopy. Each hematoxylin and eosin (H&E) stained image originates from the same sample as the confocal image directly above but not from the exact same location in the tissue

Interestingly, although a significant difference in tissue lactate fractional enrichment coming from $[1,6-^{13}\text{C}_2]\text{glucose}$ was not observed, the overall rate of lactate efflux in culture was significantly higher for primary Gleason pattern 3

cancer biopsies as compared to benign biopsies. This was presumably because of not only the trend toward higher lactate pool size (Figure 4), but also the propensity of the cancer tissue to export the lactate (Figure 2). This is consistent with prior data showing overexpression of the lactate dehydrogenase-A (LDHA) isoform (favoring lactate production from pyruvate) as well as overexpression of the monocarboxylate transporter MCT4, favoring lactate efflux, in human prostate cancer tissue compared to benign prostatic tissue.¹⁰ Moreover, prior studies have shown that both increasing expression of MCT4 mRNA as well as MCT4 protein (quantified via immunohistochemistry) correlate with higher Gleason score, suggesting that lactate efflux from prostate biopsies would provide a sensitive assessment of aggressive disease.³⁰⁻³³

In this study, a longer duration of $[1,6-^{13}\text{C}_2]\text{glucose}$ labeling was used in culture to yield a medium $[3-^{13}\text{C}]\text{lactate}$ peak with higher SNR. It was therefore important to demonstrate that the rate of lactate efflux did not change over time, and in this study the lactate efflux rate was not significantly different at late (2–26 h) versus early (0–2 h) time points (Figure 3). Theoretically, it would also be possible to increase the lactate concentration (and hence lactate SNR) effluxed into the media by decreasing the medium volume that is used during culture. However, we used a rotary tissue culture system that relies on a 2.5 mL medium volume within a 6-well plate to provide optimal exposure to medium and 5% $\text{CO}_2/21\% \text{O}_2$ air. In our experience (data not shown), alternatives such as 24-well plates do not yield the same level of tissue viability and would need to be further optimized to provide the correct ratio of medium to air exposure over time. It is also

possible to increase the SNR of dilute NMR metabolites via lyophilization³⁴; however, using the presented protocol, we achieved sufficient media lactate SNR without additional sample processing.

The fractional enrichment of C4 glutamate coming from [1,6-¹³C₂]glucose in the biopsies studied is an indication of TCA cycle activity during rotary biopsy culture (Figures 1 and 5). In the current work, we obtained a C4 glutamate fractional enrichment of 24%. This value should be viewed as a balance between TCA cycle metabolism of [1,6-¹³C₂]glucose as well as uptake of compounds that can contribute to the unlabeled tissue glutamate pool such as glutamine and glutamate. The culture medium used for this study contained 1 mM glutamine and 0.1 mM glutamate (Supporting Information).

In this study, the biopsy metabolite levels and lactate efflux rates were normalized to wet tissue weight, which could be suboptimal in studies of highly cellular tumors. In our data set, we observed a range of tissue cellularity for both benign samples (Figures 1F and 6, left) and cancer samples (Figure 6, right). As determined by a board-certified genitourinary pathologist, in benign samples, the average percentage of glandular tissue was 17%, whereas in cancer samples, the average percentage of glandular tissue was 9%, and the average percentage of cancer was 12%. In future studies with a higher number of samples, we intend to fit a multivariate model of lactate production as a function of the predictor variables of cancer percentage and benign glandular percentage.

The choice of an appropriate metabolite normalization standard is an ongoing challenge in metabolic studies of heterogeneous tissues, as opposed to cells. In studies of cultured cells, it is common to normalize metabolites to the number of cells or to a metric of cell mass such as total protein. In tissues, automated cell counting and/or protein quantification requires tissue digestion, making it impossible to obtain high-quality histopathology. It is possible to estimate tissue cellularity using automated image analysis of histopathology slides³⁵; however, given the heterogeneity of human prostate tissue, this approach might yield inaccurate results unless the fixed, embedded, and stained tissue is comprehensively sliced to yield a 3D image stack. Another possibility is to analyze the metabolites of interest in terms of their ratios to a reference metabolite.^{10,36,37} This can be a robust method, but it does yield a metric that is sensitive to the reference metabolite as well as the metabolite of interest. Therefore, each normalization technique has strengths and limitations to practical implementation.

This study had a number of limitations. Our goal was to develop the biopsy culturing and metabolic profiling protocol, so we focused on biopsies routinely acquired from patients undergoing active surveillance or workup for prostate-related symptoms who had not been previously treated. Because of the low prevalence of aggressive cancer in this population, we only obtained biopsies containing benign

glands or primary Gleason pattern 3 cancer, which limited our ability to further characterize the metabolic phenotype of aggressive prostate cancer.

5 | CONCLUSION

In summary, a protocol was developed that allowed the quantification of lactate production and efflux in single living human prostate biopsies (1–6 mg of tissue), as well as the potential to quantify TCA metabolism through the quantification of fractional enrichment of glutamate. This was accomplished by optimizing experimental parameters such as the duration of time in rotary tissue culture (24 h) and the parameters of the NMR acquisition to achieve sufficient SNR from small metabolite signals within a 3 h and 25 min HR-MAS acquisition time. Validation studies demonstrated that this optimized protocol provided measurements of lactate production and/or fractional enrichment and efflux consistent with prior data describing lactate pool size, LDHA, and MCT4 expression in benign human prostatic tissue versus prostate cancer.^{4,10} Having established this protocol, similar measurements can then be performed on treatment-resistant and/or metastatic prostate cancer biopsies for a better understanding of metabolic perturbations in the setting of aggressive prostate cancer.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

TABLE S1 Culture medium formulation

TABLE S2 Sources of culture medium ingredients

TABLE S3 Biopsy tissue NMR measurements: automated HR-MAS shimming routine

TABLE S4 Biopsy media NMR measurements: automated shimming routine

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