



h13C MRI Detects Stimulation of Distal Tumor Glycolytic Flux From Hepatic Ablation in a Breast Metastasis Model

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$h^{13}C$ MRI Detects Stimulation of Distal Tumor Glycolytic Flux from Hepatic Ablation in a Breast Metastasis Model

by

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**Submitted in Partial Fulfillment of the Requirements for the M.D. Degree
with Honors in a Special Field at Harvard Medical School**

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Statement of significance: In vivo h13C MRI may be able to detect adverse changes in tumor behavior with greater sensitivity than conventional imaging, allowing for greater precision in cancer management.

Abstract

Thermal ablation is an effective therapy for liver tumors, however there is evidence that it can stimulate tumor growth at distant sites via the HFG/c-Met pathway, which is also linked to tumor metabolism. We evaluated this effect *in vivo* using hyperpolarized ^{13}C -pyruvate MRI.

Fisher rats were implanted with R3230 rat breast adenocarcinoma cells (n=28) and assigned to either: sham surgery, hepatic radiofrequency ablation (RFA), or hepatic RFA + adjuvant c-Met inhibition with PHA-665752 (single IP dose, 0.83 mg/kg at 24h post-RFA). MRI was performed 24h before and 72h after treatment, and the conversion of ^{13}C -pyruvate into ^{13}C -lactate within each tumor was quantified as lactate:pyruvate ratio (LPR).

Hepatic RFA alone resulted in increased growth of the distant tumor compared to sham (0.50 ± 0.13 mm/day and 0.11 ± 0.07 mm/day, respectively; $p < 0.0001$). In the hepatic RFA + adjuvant PHA group, tumor growth rate declined immediately following PHA administration, with a post-treatment growth rate (0.06 ± 0.13 mm/day) similar to the sham group ($p = 0.28$). A significant increase in LPR was seen following hepatic RFA ($p = 0.02$), while LPR was unchanged in the sham ($p = 0.10$) and RFA+PHA ($p = 0.90$) arms.

In vivo $h^{13}\text{C}$ -pyruvate MRI can detect hepatic RFA-induced increases in lactate conversion within a distant R3230 tumor, which is coupled with relatively increased tumor growth. Adjuvant inhibition of c-Met suppresses these off-target effects. The results highlight the potential for $h^{13}\text{C}$ -pyruvate MRI as a potential *in vivo* tool to detect lesions at risk for off-target RFA stimulation and suggest a possible role for adjuvant c-Met inhibition in such cases.

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Glossary

$h^{13}C$ MRI: Hyperpolarized ^{13}C Carbon Magnetic Resonance Imaging

RFA: Radiofrequency ablation

HCC: Hepatocellular carcinoma

HGF: Hepatocyte growth factor

c-Met: receptor tyrosine kinase, hepatocyte growth factor receptor

HIF: Hypoxia-inducible factor

FDG-PET: Fluorodeoxyglucose-positron emission tomography

PHA: specific c-Met inhibitor PHA-665752

R3230: c-Met positive rat breast adenocarcinoma cell line

LPR: Lactate:pyruvate ratio

RARE: Rapid acquisition with refocused echo

EPSI: Echo planar spectroscopic imaging

ROI: Region of interest

RECIST: Response evaluation criteria in solid tumors

Introduction

Hepatic radiofrequency thermal ablation (RFA) can stimulate distant tumor growth

Thermal tumor ablation using radiofrequency energy (RFA) is a widely used therapeutic tool for the treatment of many types of focal tumors including hepatocellular carcinoma (HCC) and metastases from breast and colorectal cancers, with clinical outcomes comparable to surgical resection in well-selected patients.¹⁻³ Indeed, based on proven efficacy especially for small, focal lesions, RFA has become a vital component of oncologic therapy today and has been incorporated into a number of clinical treatment guidelines. However, there is increasing clinical and experimental evidence that hepatic RFA may have unintended effects such as inducing tumor growth and more aggressive tumor biology both within and outside of the liver.³⁻⁶ For example, in a study of 580 patients with small (<3 cm) HCC tumors, RFA led to a 25% higher 5-year cumulative distant intrahepatic new tumor rate (62.7% vs. 36.6%) and worse disease-free survival rate (31.7% vs. 61.1%) compared to surgical resection.⁷ Similar effects of RFA, including higher long-term rates of new liver tumors compared to epidemiologic data of those not treated with RFA, have been shown in other studies in HCC and colorectal metastases.^{3,8-12} Other ablation techniques, such as thermal ablation with laser or microwave have shown similar effects.¹³ These off-target pro-oncogenic effects following hepatic thermal tumor ablation could have a major impact on the treatment algorithm of patients with multifocal disease or those who are at high risk for metastases.

HGF/c-Met Pathway is stimulated by RFA of normal liver

The hepatocyte growth factor (HGF) and corresponding receptor tyrosine kinase cell surface receptor c-Met have been implicated as a pathway of distal tumor stimulation by liver RFA.^{5,14} The HGF/c-Met pathway is vital for proper liver development and regeneration, but is frequently deregulated and overexpressed in many human malignancies.^{15,16} RFA of normal rat liver stimulates HGF and c-Met expression in the region immediately surrounding the ablation zone, termed the periablational zone, as well as in distant tumors.¹⁴ This activation of the HGF/c-Met pathway stimulates growth and proliferation of distant c-Met-positive tumors.¹⁴ This effect is not demonstrated in c-Met-negative tumors or when an adjuvant c-Met inhibitor is combined with RFA for c-Met-positive tumors.¹⁴ These results support the role of c-Met inhibition as a potential option to suppress unintended off-target tumor stimulatory effects of hepatic RFA. Yet, identifying those tumor types or patients who are at higher risk for c-Met-mediated off-target tumor stimulation following thermal ablation remains a clinical challenge.

HGF/c-Met regulates tumor glycolysis

HGF binding to c-Met activates a series of signaling pathways (such as PI3K/Akt, Src, MAPK/Erk, STAT3, and FAK) involved in tumor cell migration, proliferation, survival, metastasis, and angiogenesis.^{15,16} Upregulation of tumor glycolysis, a hallmark feature of cancer cells also known as the Warburg Effect, has been attributed to the downstream PI3K/Akt and Akt/mTOR pathways in several different cancers, including HCC.^{17–21} Further downstream is hypoxia-inducible factor (HIF), which induces angiogenesis and promotes glycolysis, and is overexpressed at the periablational zone.⁴

Detection of HGF/c-Met Tumorigenesis with Hyperpolarized ^{13}C -pyruvate/lactate MRI

Hyperpolarized ^{13}C MRI ($h^{13}\text{C}$ MRI) provides a non-invasive technique for acquiring high-resolution imaging of the uptake and metabolism of ^{13}C -containing tracer molecules in real-time.^{22,23} $h^{13}\text{C}$ -pyruvate MRI detects conversion into $h^{13}\text{C}$ -lactate, providing an imaging biomarker for the last step in glycolysis. While this technique probes a number of factors linked to rates of glycolysis, including perfusion, monocarboxylic acid transporter activity, and LDH activity, tumor lactate production is of particular interest because it is elevated in many tumors and is a known marker of cancer proliferation and aggressiveness.^{22,24} Current imaging surveillance techniques, which focus mainly on size, may fail to catch at-risk lesions with such a metabolic profile. Even FDG-PET, a staple of nuclear medicine imaging for analyzing metabolic activity, is unable to discriminate between the causes of increased glucose uptake, resulting in similar signal in both tumors and surrounding metabolically active tissues such as muscle.²⁵ However, $h^{13}\text{C}$ MRI offers another layer to further differentiate by type of metabolic activity, specifically by allowing direct analysis of the Warburg Effect that is prevalent in many types of cancers.²⁵

In this study we used $h^{13}\text{C}$ -pyruvate/lactate MRI to quantify tumor lactate production in a rat model of metastatic c-Met-positive adenocarcinoma. We compared lactate production and growth of these subcutaneous-based tumors between a control group and in rats subjected to RFA of normal liver. We hypothesized that in rats treated with liver RFA, increased HGF/c-Met activity would lead to increased tumor growth and lactate production. We tested the dependency of this effect on c-Met activation by performing $h^{13}\text{C}$ MRI and tumor growth

measurements on a third cohort of rats who underwent both liver RFA and treatment with a specific c-Met inhibitor, PHA-665752.

Materials and Methods

Experimental Design

This study was approved by our institutional animal care and use committee (IACUC). Female Fischer rats (Charles River Laboratories, Wilmington, MA) were implanted with R3230 adenocarcinoma cells subcutaneously (n=28). Tumor growth was measured daily throughout the experiment using calipers. When tumors reached 10-12 mm in diameter (Day 0), animals were randomly assigned to one of three treatment arms: sham (laparotomy without RFA), RFA (RFA to the left hepatic lobe), or RFA+PHA (RFA of the left hepatic lobe plus adjuvant c-Met inhibition with PHA-665752). To limit variations in pre-treatment tumor growth between treatment groups, tumors were screened before treatment and only those demonstrating similar pre-treatment growth rates and size were used. Animals in which tumors did not grow or did not reach the threshold of 10-12 mm were excluded. A total of 9, 13, and 6 rats met these inclusion criteria for the sham, RFA, and RFA+PHA arms, respectively.

Hyperpolarized ^{13}C -pyruvate/lactate MRI was performed 24 hours prior to treatment and 72 hours post-treatment on a random subset of rats from each arm, totaling 5, 5, and 4 in the sham, RFA, and RFA+PHA arms, respectively. A fifth rat within the RFA+PHA arm died prematurely before imaging could be completed. Relative quantities of ^{13}C -pyruvate and ^{13}C -lactate were measured within each intratumoral voxel and reported as lactate:pyruvate ratios (LPR). All animals were sacrificed at 7 days post-treatment. All experiments were performed by

individuals with experience in performing tumor implantation, RF ablation, and surgery in these models.

Animal Tumor Model

The R3230 adenocarcinoma cell line is a well-characterized c-Met positive cell line with established tumor growth rates.^{26–28} R3230 xenografts were implanted into the mammary fat pad of female Fisher rats (120-150 g) via subcutaneous injection of 300 μ L of tumor suspension containing 10^7 cancer cells for each animal.

RFA and Sham treatments

Anesthesia was induced using isoflurane in oxygen (5% for induction, followed by 1-3% for maintenance) for all surgeries. The left hepatic lobe was exposed via surgical laparotomy with a subcostal incision. The 1-cm-tip of a 21-gauge insulated electrode was placed on the normal left hepatic lobe parenchyma, with the animal resting on a standardized metallic grounding pad. For the RFA and RFA+PHA groups, a monopolar RF ablation generator (500kHz RF generator, Radionics, Model CC-1) was used to energize the tip for 5 minutes, with output titrated to maintain tip temperatures of $70^{\circ}\text{C} \pm 2$. For the sham treatment group, the left hepatic lobe was exposed for 5 minutes, but no RFA was performed. Following treatment, the incision was closed with 4-0 Vicryl and 4-0 Prolene sutures and a single SC dose of Buprenorphine (0.2 mg/kg) was administered for perioperative comfort.

Tumor Growth Measurements

Tumors were measured along both longitudinal and transverse diameters using mechanical calipers, and an average diameter was calculated. All tumor measurements reported throughout this manuscript refer to such average diameters. Measurements began three days after tumor implantation, when tumors were typically palpable. Subsequently, tumor size was measured daily. Once tumors reached 10-12 mm (Day 0), animals were assigned to one of the three treatment arms. To control for confounding due to differences in tumor growth between treatment arms, animals were matched according to pre-treatment growth rate and size, with similar animals spread into different treatment arms. Size measurements were recorded until 7 days post-treatment, then the animals were sacrificed.

c-Met Inhibitor (PHA) Administration

The c-Met inhibitor PHA-665752 was obtained in powder form and mixed with 0.9% NaCl. The PHA was administered in a single 0.83 mg/kg intraperitoneal dose 24 hours post-RFA to the “RFA + adjuvant c-Met inhibitor” (“RFA + PHA”) arm only, as per prior studies.¹⁴

¹H and Hyperpolarized ¹³C-pyruvate MRI

MRI was performed on a 9.4 T horizontal-bore small-animal MRI system (Bruker Biospec 94/20, Billerica MA) with an 84mm quadrature proton coil and an in-house built 27mm transmit/receive ¹³C surface coil. During imaging, animals were anesthetized with isoflurane in oxygen (5% for induction, followed by 1-3 % for maintenance), and the level of anesthesia was

assessed by respiratory monitoring. Thermal support was provided by a warm-air circulator with fiber-optic temperature feedback.

Axial and coronal T2-weighted anatomical images were acquired using a rapid acquisition with refocused echo sequence (RARE) with a field of view of 6 cm, 128 x 128 matrix, echo train length of 8, TR 1245 ms, TE 11 ms, 2 mm slice thickness.

Hyperpolarized ^{13}C -pyruvate solution was prepared by dynamic nuclear polarization (DNP) using a commercial DNP polarizer (Hypersense, Oxford Instruments Molecular Biotools, Oxfordshire UK). $[1\text{-}^{13}\text{C}]$ pyruvic acid was combined with 15mM OX063 radical (GE Healthcare, London UK) and 1mM gadoteridol (ProHance, Bracco, Milan Italy) and polarized for 40 minutes or more at 1.4K and 100mW microwave power. The polarized material was then dissolved in saline containing 50mM TRIS and 125mg/l EDTA and adjusted to physiological pH with sodium hydroxide to obtain 96mM hyperpolarized pyruvate solution for injection. 2.5ml of this solution were administered as a 10-15s bolus starting a few seconds after the initiation of the ^{13}C MRSI acquisition. ^{13}C data were acquired in the coronal plane across the tumor using an echo planar spectroscopic imaging (EPSI) sequence with a field of view of FOV 6 cm, 4° tip angle, 16x16 matrix, 10mm slice thickness, 512 spectral points, and 4kHz spectral width. 64 repetitions were acquired with 2s temporal resolution.

Image processing was performed in Mathematica (Wolfram), and ImageJ (NIH). Spectroscopic image data were separated into even and odd echoes, apodized with a 30Hz line-broadening filter, and reconstructed with standard Fourier methods to obtain pixel-by-pixel spectra with 2kHz spectral width. Spectra from even and odd echoes were then combined after application of a first-order phase to account for the temporal offset between the starting points

of the two echo trains. Pixel-by-pixel magnitude spectra were computed, and pyruvate and lactate signals were quantified by integrating their spectral peaks, after subtracting the mean magnitude noise from the spectra to eliminate spurious baseline signals. Magnitude noise was estimated from a noise-only spectrum. Lactate-to-pyruvate ratios were computed using the time integrals of each metabolite's signal, a measure that has been shown to reflect the rate of label exchange between pyruvate and lactate.²⁹ Lactate production was reported as the lactate:pyruvate ratio (LPR) to account for intertumoral variations in ¹³C-pyruvate perfusion. The T2-weighted ¹H coronal images were used to define a tumor region-of-interest (ROI), and LPR was obtained by averaging all intratumoral voxels contained within this tumor ROI. Example ¹H and ¹³C MRI are shown in Figure 1.

Data Reporting and Statistical Analysis

Prism (GraphPad Software, San Diego, CA) was used for statistical analysis. All data are reported as means $\pm$ standard deviations. Tumor growth curves before and after treatment were analyzed with linear regression analysis models to determine the slope of the pre- and post-treatment growth curve on a per-tumor basis. Average pre- and post-treatment growth rates (slope) could then be calculated for each treatment arm. Changes in LPR (Δ LPR) were calculated by simply subtracting pre-treatment LPR from post-treatment LPR for each tumor, from which averages could be calculated for each treatment arm. A two-sampled t-test was used with a significance criterion of 0.05 and power of 0.80 to estimate minimal sample sizes. We placed conservative estimates for standard deviation (0.05) and the difference in mean for LPR (0.1),

based on measurements from preliminary imaging, yielding a minimum sample size of 4 tumors per arm.

When comparing pre-treatment growth rates with post-treatment growth rates within the same treatment arm, a two-sided paired t-test was used. For all statistical analyses in this study, when comparing values between different treatment arms, a two-sample unpaired t-test was used. A one-sample t-test was used to determine if changes in LPR within one treatment arm were significant (i.e. null hypothesis = no change). $p < 0.05$ was considered statistically significant.

Results

Tumors in the RFA arm demonstrate relatively increased growth

Growth curves for distant tumors in the three study arms are shown in Figure 2A, and data on tumor growth are summarized in Table 1. Tumor growth rates and tumor diameter prior to treatment were not significantly different for all comparisons between treatment arms. The sham treatment group exhibited a decrease in tumor growth rate following treatment (pre-treatment: 0.59 ± 0.14 mm/day; post-treatment: 0.11 ± 0.07 mm/day; $p < 0.0001$). In the RFA group, the rate of growth of the distant tumor did not change significantly (pre-treatment: 0.58 ± 0.15 mm/day; post-treatment: 0.50 ± 0.13 mm/day; $p = 0.20$). The tumors in the RFA+PHA group responded similarly to the sham group, with a decrease in growth rate following treatment (pre-treatment: 0.50 ± 0.18 mm/day; post-treatment: 0.06 ± 0.13 mm/day; $p = 0.004$). This resulted in a tumor end diameter for the RFA only group (14.5 ± 1.2 mm) that was significantly greater than that of the sham (12.1 ± 0.8 mm; $p < 0.0001$) and RFA+PHA (11.3 ± 1.3 mm; $p = 0.0003$) groups.

Tumors in the RFA+PHA arm were not significantly different in size from sham at the time of sacrifice ($p=0.22$).

Post-treatment tumor growth rates for the different treatment groups are shown in Figure 2B. Hepatic RFA alone resulted in relatively increased growth of the distant R3230 tumors (post-treatment growth rate of 0.50 ± 0.13 mm) compared to sham treatment (0.11 ± 0.07 mm; $p<0.0001$). In the RFA+PHA group, tumors exhibited a post-treatment growth rate (0.06 ± 0.13 mm) that was significantly less than that of the group receiving RFA only ($p<0.0001$), and similar to that of the sham group ($p=0.28$).

Tumors in the RFA arm demonstrate relatively greater lactate production

The change in lactate:pyruvate ratio before and after treatment for all individual tumors in each treatment group is shown in Figure 3A. Hepatic RF ablation alone resulted in a unanimous increase in distant tumor LPR. Response to treatment was more variable in animals also given adjuvant c-Met inhibition (PHA). Figure 3B shows the average change in LPR before and after treatment for each treatment group. Hepatic RFA results in a significant increase in LPR (0.02 ± 0.01 ; $p=0.02$), or a mean increase of $13 \pm 8\%$, coupled with the relatively increased tumor growth shown previously. There is no significant change in LPR for the RFA+PHA (0.00 ± 0.04 ; $p=0.90$) or sham (-0.05 ± 0.05 ; $p=0.10$) groups. Δ LPR for the RFA group was significantly greater in comparison to the sham group (unpaired t-test $p=0.03$). LPR calculations are summarized in Table 2.

In order to determine whether these changes in LPR could be due simply to changes in tumor size, we plotted tumor size vs. LPR at the time of pre-treatment imaging (Day -1) for all

arms, before treatment could have had an effect on tumor metabolism. There is a significant negative correlation (Pearson correlation: $r = -0.6394$ [95% CI -0.8736 to -0.1648], $r^2 = 0.4089$, $p = 0.0138$) whereby increased tumor size is correlated with decreased LPR. Average pre-treatment LPR for the RFA only arm is significantly less than that of the RFA+PHA group ($p = 0.02$), while there is no significant difference between sham vs. RFA ($p = 0.06$) or sham vs. RFA+PHA ($p = 0.53$).

Discussion

In this study, $h^{13}C$ -pyruvate MRI demonstrated a significant increase in lactate production within distant subcutaneous R3230 tumors following liver RFA, in agreement with other studies showing upregulation of tumor glycolysis with stimulation of the HGF/c-Met axis.^{19,20} Hepatic RFA results in not only an increase in lactate production, but also relatively increased tumor growth. This off-target effect is suppressed with c-Met inhibition. This is the first study demonstrating *in vivo* detection of this effect on glycolysis with MRI.

The effects of hepatic RFA on distant tumor growth are consistent with those of Ahmed et al. (2015), which linked these changes to the central role of the HGF/c-Met signaling axis.¹⁴ This study represents a further extension of this investigation by adding $h^{13}C$ MRI data to link the HGF/c-Met signaling axis to measurable changes in *in vivo* tumor metabolism. The results show the potential of $h^{13}C$ MRI as a noninvasive method to potentially identify patients at risk for increased tumorigenesis following local thermal ablation therapies, as well as to monitor response to c-Met inhibition, which could be used to prevent or reduce unintended off-target effects. $h^{13}C$ MRI may also offer a method to monitor therapeutic response more broadly, potentially to a wide range of targeted therapies and perhaps before changes in tumor size are

evident on traditional imaging modalities. The potential for a non-invasive biomarker that is more sensitive and accurate than size-based measurements is particularly appealing given the increasing popularity of immunotherapies, which have highlighted limitations in RECIST-based monitoring.³⁰ There is close coupling between angiogenesis, hypoxia, and glycolysis via HIF-1 α and PI3K downstream of c-Met, all likely contributing to the increase in tumor LPR in the hepatic RFA arm of our study, and requiring future studies to elucidate the underlying mechanisms. With many pathways converging to modulate the metabolic activity of tumors, h¹³C MRI is uniquely suited to analyze response to a wide range of therapies through analysis of a common end-product.

There was tapering of the tumor growth rate seen in the sham and RFA+PHA groups following treatment. This has been seen on prior studies, and we attribute this to an immune response likely stimulated by laparotomy. Other tumor models also demonstrate this effect, which can even lead to complete regression.³¹ We took care to standardize all surgical procedures, including administration of anesthesia and post-surgical analgesics, to minimize variability from this effect. While this could be avoided by using an immunodeficient model, we believe that immunocompetent animals were necessary for a more accurate model in this case, as inflammatory markers such as interleukin-6 have been linked to off-target RFA oncogenic effects.³² The underlying inflammatory pathways that influence glycolytic activity and lactate flux as detected by h¹³C MRI is an area for further investigation.

Compared to the growth curve analysis, a smaller number of animals underwent h¹³C MRI. This was mostly due to logistical limitations, as the imaging studies could be performed only within a particular window based on tumor growth and magnet availability. There was also a

premature death in the RFA+PHA arm before the post-treatment $h^{13}C$ MRI data could be acquired, which resulted in one less rat in that arm than intended. Nevertheless, this did not impact our results, which showed a clear consistent increase in LPR within the RFA group only, while mixed results were already seen in the RFA+PHA arm and this would not have changed even with the inclusion of an additional rat.

Even when using growth-rate-matched tumors there was variability of the baseline LPR values seen across all three arms. This heterogeneity is likely influenced by many factors, for example variations in the general metabolic state of each animal or the heterogeneity of the tumor microenvironment across different animals. We took care to minimize variations related to anesthesia and body temperature maintenance during the imaging experiments. Furthermore, animals were selected into treatment arms at random, and while the tumors were grown in 4-10 animals at a time, the treatment arms were not grouped together but were instead performed in randomized order to minimize systemic errors related to the timing of the experiments. We also controlled for these potential variations by focusing on the net change in LPR rather than on absolute values. Despite these initial variations in LPR that may represent baseline variations in glycolytic activity, there was still a clear net increase in LPR demonstrated in the RFA group, further supporting the conclusion that distant R3230 tumor lactate production was stimulated by hepatic RFA.

One limitation to this study was the inability to directly correlate $h^{13}C$ MRI with tumor c-Met expression levels, as we were also correlating $h^{13}C$ MRI to growth curves and thus could not harvest the tumors at the time of imaging. Furthermore, the comparison of pre-treatment and post-treatment LPR was more informative than a single LPR measurement, and it would not have

been possible to perform both *in vivo* imaging and *ex vivo* analyses at two time points for each tumor. However, the R3230 model, pre-treatment growth rate matching, PHA dosing, and RFA time and energy settings in this study matched that of the Ahmed et al. study, which showed that tumors in the RFA-treated group demonstrated an increase in c-Met expression 3 days post-treatment¹⁴, matching the timing of post-treatment $h^{13}C$ MRI in this study. A more direct verification of this link between changes in c-Met expression and lactate flux as measured by $h^{13}C$ MRI would require *in vivo* sampling, which would be technically difficult and may directly change the tumor metabolism and introduce artifacts on the post-treatment MRI.

The negative correlation between pretreatment tumor size and LPR is not well understood. It is possible that the stroma overlying the tumor may be a contributor, as this may influence vascularity and perfusion, which can subsequently influence glycolytic activity. We attempted to control for such differences by matching pre-treatment tumor growth rates, as previously discussed. Nevertheless this observation increases our confidence that the increase in LPR seen in the hepatic RFA arm is in fact due to an RFA-induced effect, as the observed negative correlation between tumor size and LPR would otherwise suggest an opposite effect, where an increase in tumor size would correspond to a net decrease in LPR. Due to the lack of significant differences in pre-treatment tumor size or growth rate between all arms, this correlation cannot explain the difference in pre-treatment absolute LPR values seen between the RFA and RFA+PHA arms.

Conclusion

In vivo $h^{13}C$ -pyruvate MRI showed increased lactate conversion within a distant metastatic tumor model following liver thermal ablation therapy, coupled with relatively increased tumor growth. Adjuvant c-Met inhibition suppressed both distant tumor growth and lactate production, supporting a role for the HGF/c-Met signaling axis in this tumorigenic response. $h^{13}C$ MRI may potentially offer a new non-invasive *in vivo* tool in the management of patients undergoing thermal ablation therapies.

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Tables and Figures

Table 1: Summary of Distant Subcutaneous Tumor Growth

Treatment Arm	Pre-Treatment Growth Rate (mm/day)	Post-Treatment Growth Rate (mm/day)	Tumor End Diameter (mm) [†]	Change in Tumor Diameter after Treatment (mm) [†]
Sham (n=9)	0.59 ± 0.14	0.11 ± 0.07	12.1 ± 0.8	1.1 ± 0.6
RFA only (n=13)	0.58 ± 0.15	0.50 ± 0.13*	14.5 ± 1.2*	3.5 ± 1.0*
RFA + PHA (n=6)	0.50 ± 0.18	0.06 ± 0.13	11.3 ± 1.3	0.7 ± 1.3

Note: Data are means ± standard deviations.

** $p < 0.05$ when compared to the sham group.*

[†] Analysis excludes animals who did not survive through Day 7 (2 animals in RFA only group, 1 animal in RFA+PHA group)

Table 2: Summary of Distant Subcutaneous Tumor Lactate Production (lactate:pyruvate ratio, or LPR)

Treatment Arm	Pre-Treatment LPR	Post-Treatment LPR	ΔLPR
Sham (n=5)	0.19 ± 0.06	0.14 ± 0.02	-0.05 ± 0.05
RFA only (n=5)	0.13 ± 0.03	0.14 ± 0.02	0.02 ± 0.01*
RFA + PHA (n=4)	0.22 ± 0.06	0.22 ± 0.07	0.00 ± 0.04

Note: Data are means ± standard deviations.

** Denotes a statistically significant change in LPR ($p < 0.05$).*

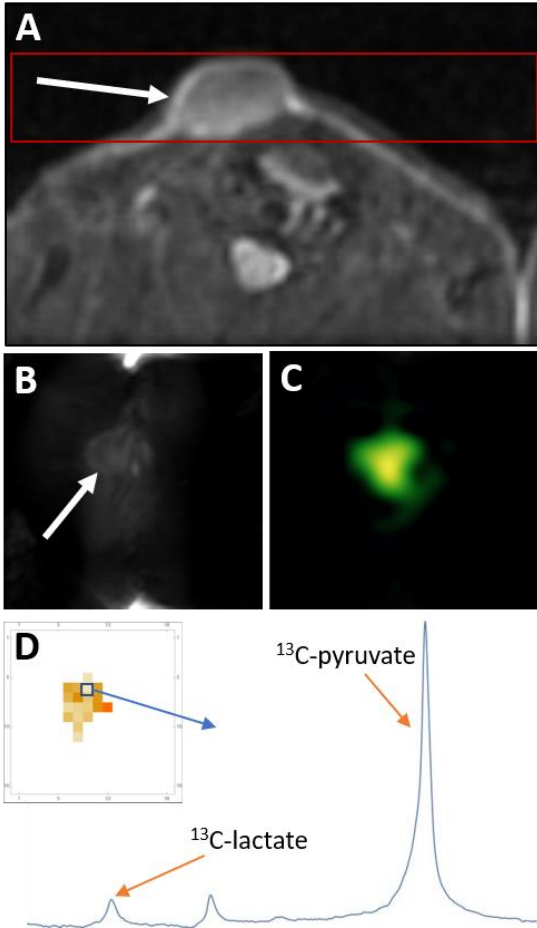


Figure 1: Example MRI images of an R3230 tumor. T2-weighted ^1H axial (A) and coronal (B) images of a tumor, denoted by arrows. Red lines denote the coronal slice used for ^{13}C MRI. C) ^{13}C -lactate image showing ^{13}C -lactate production within the tumor (zero-fill interpolated to a 128×128 matrix). D) ^{13}C -lactate and ^{13}C -pyruvate spectra from a tumor voxel, with inset showing location within a non-interpolated LPR tumor ROI map.

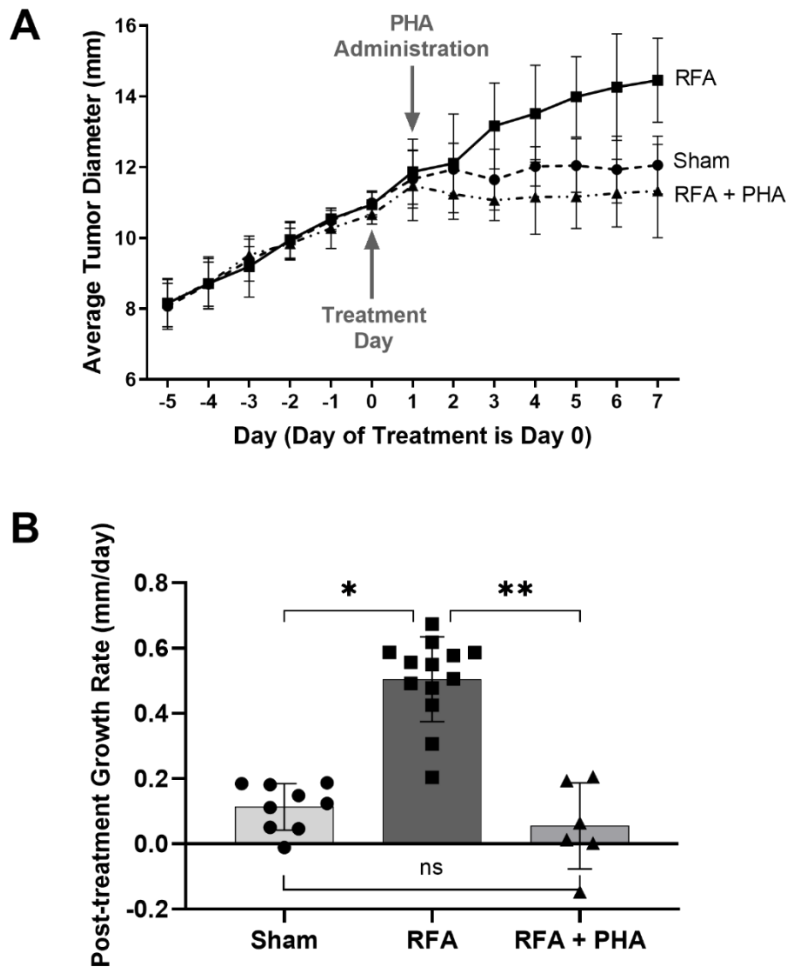


Figure 2: Hepatic RFA induces distant subcutaneous R3230 tumor growth compared with sham treatment, while adjuvant c-Met inhibition (PHA) suppresses this effect. All error bars represent standard deviations. **A)** Tumor growth curves show the same rate of growth of all arms prior to treatment. Upon treatment, tumor growth in the sham group begins to slow, while the RFA group tumors continue to grow to an end diameter that is significantly greater than that of the Sham group ($p < 0.0001$). Adjuvant c-Met inhibition (PHA) leads to slowing of tumor growth similar to sham, with an end diameter smaller than the RFA group ($p = 0.0003$) and similar to Sham ($p = 0.22$). **B)** Symbols represent post-treatment growth rates for each individual tumor, while bars show average values per treatment group. Average tumor growth rate following RFA is greater in comparison to the Sham (*: $p < 0.0001$) and RFA+PHA (**: $p < 0.0001$) arms. Adjuvant c-Met inhibition (RFA+PHA) results in a post-treatment growth rate that is not significantly different from that of the sham treatment arm ($p = 0.28$).

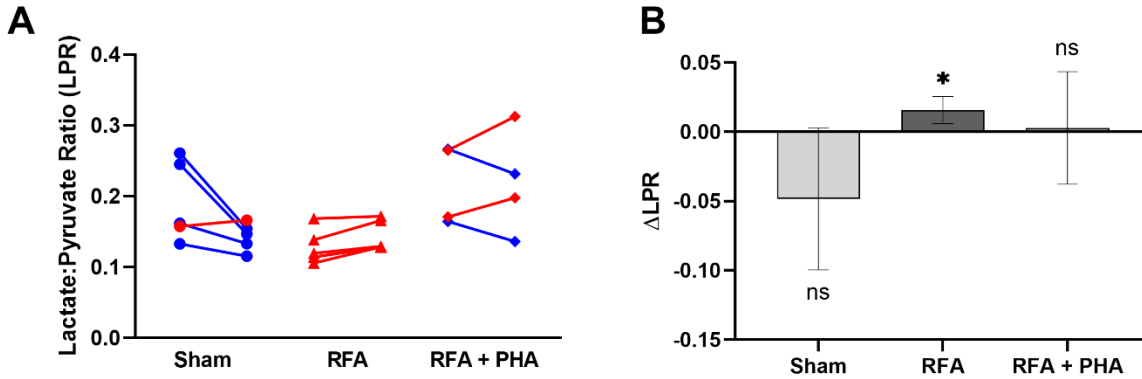


Figure 3: Hepatic RF ablation results in upregulation of lactate production in distant subcutaneous R3230 tumors, as seen by increasing lactate:pyruvate ratio (LPR). **A)** The graph shows LPR trends for all individual tumors in each treatment group, with lines connecting pre-treatment LPR and post-treatment LPR. Red lines denote an increasing LPR, and blue lines denote a decreasing LPR. While sham group tumors overwhelmingly exhibit decreasing LPR, the RFA group tumors show unanimously increasing LPR. The addition of adjuvant c-Met inhibition (PHA) results in a more variable response. **B)** The graph shows the average change in LPR before and after treatment for each treatment arm. The significant increase in LPR seen following hepatic RF ablation (*: $p=0.02$) is suppressed with adjuvant c-Met inhibition ($p=0.90$). The decrease in LPR seen in the Sham group is not statistically significant ($p=0.10$). Error bars represent standard deviations.

Manuscript for Publication

The previously presented manuscript represents a revised version of the manuscript “h13C MRI Detects Stimulation of Distal Tumor Glycolytic Flux from Hepatic Ablation in a Breast Metastasis Model” that was submitted to the peer-reviewed medical journal *NMR in Biomedicine* on August 30, 2019. The manuscript underwent peer review, and the authors have been invited to resubmit a revised manuscript and responses to reviewer comments. This manuscript represents the revised version that will be resubmitted before March 3, 2020 for potential publication.