

# Preclinical evaluation of <sup>11</sup>C-sarcosine as a substrate of proton-coupled amino acid transporters and first human application in prostate cancer

Morand Piert<sup>1</sup>

Xia Shao<sup>1</sup>

David Raffel<sup>1</sup>

Mathew S. Davenport<sup>1</sup>

Jeffrey Montgomery<sup>2</sup>

Lakshmi Priya Kunju<sup>3</sup>

Brian G. Hockley<sup>1</sup>

Javed Siddiqui<sup>3,4</sup>

Peter J. H. Scott<sup>1</sup>

Arul M. Chinnaiyan<sup>3,4</sup>

Thekkelnaycke Rajendiran<sup>3,4</sup>

## Authors Affiliations:

<sup>1</sup> Department of Radiology, University of Michigan, Ann Arbor, MI, 48109, USA

<sup>2</sup> Department of Urology, University of Michigan, Ann Arbor, MI, 48109, USA

<sup>3</sup> Pathology Department, University of Michigan, Ann Arbor, MI, 48109, USA

<sup>4</sup> Michigan Center for Translational Pathology, University of Michigan, Ann Arbor, MI, 48109,

USA

**Corresponding author:**

Morand Piert, M.D.,

Professor of Radiology

University of Michigan Health System

Department of Radiology, Division of Nuclear Medicine

University Hospital B1G505C

1500 E. Medical Center Drive

Ann Arbor, MI 48109-0028, USA

Tel. (734) 936 5388

FAX. (734) 936 8182

Email: mpiert@umich.edu

**Running title:** Novel  $^{11}\text{C}$ -sarcosine PET tracer

**Keywords:**  $^{11}\text{C}$ -sarcosine, prostate cancer, proton-coupled amino acid transporter (PAT)

**No conflict of interest** is declared

**Word count:** 6021

## ABSTRACT

Sarcosine is a known substrate of proton-coupled amino acid transporters (PAT), which are overexpressed in selected tissues and solid tumors. Sarcosine, an N-methyl derivative of the amino acid glycine and a metabolic product of choline, plays an important role for prostate cancer aggressiveness and progression. **Methods:** Carbon-11 radiolabeled sarcosine ( $^{11}\text{C}$ -sarcosine) was tested as a new positron emission tomography (PET) imaging probe in comparison with  $^{11}\text{C}$ -choline in two prostate cancer tumor xenograft models (DU-145 and PC-3). We characterized  $^{11}\text{C}$ -sarcosine transport in PC-3 and LNCaP tumor cells and performed  $^{11}\text{C}$ -sarcosine PET with computed tomography (CT) in the first human subject with localized Gleason 4+3 prostate cancer. Target metabolite analyses of sarcosine and its natural precursors, glycine and choline, were performed from independent human prostate tissues. **Results:** In vitro assays indicated blockage of  $^{11}\text{C}$ -sarcosine uptake into PC-3 and LNCaP tumor cells by excess unlabeled (“cold”) sarcosine. 5-Hydroxy-L-tryptophan (HT), but not 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), competitively inhibited  $^{11}\text{C}$ -sarcosine tumor cell uptake, confirming PAT-mediated transport. In vivo tumor-to-background ratios (TBR) obtained from  $^{11}\text{C}$ -sarcosine PET were significantly elevated compared to  $^{11}\text{C}$ -choline in DU-145 (TBR  $^{11}\text{C}$ -sarcosine:  $1.92 \pm 0.11$  vs.  $1.41 \pm 0.13$  for  $^{11}\text{C}$ -choline ( $n = 10$ ;  $P < 0.002$ ), and PC-3 tumors (TBR  $^{11}\text{C}$ -sarcosine:  $1.89 \pm 0.2$  vs.  $1.34 \pm 0.16$  for  $^{11}\text{C}$ -choline ( $n = 7$ ;  $P < 0.002$ ).  $^{11}\text{C}$ -sarcosine produced high-contrast images in one case of localized clinically significant prostate cancer. Target metabolite analyses revealed significant step-wise increases of sarcosine, glycine and choline tissue levels from benign prostate tissue to localized prostate cancer and subsequently metastatic disease.  $^{11}\text{C}$ -sarcosine showed a favorable radiation dosimetry with an ‘effective dose’ estimate of 0.0045 mSv/MBq,

resulting in 2.68 mSv for a human subject (600 MBq dose). **Conclusion:**  $^{11}\text{C}$ -sarcosine is a novel radiotracer for PAT transporters and shows initial utility for prostate cancer imaging, with potential benefit over commonly used  $^{11}\text{C}$ -choline.

## INTRODUCTION

The prostate cancer metabolome is characterized by an increased amino acid metabolism and a perturbation of nitrogen breakdown pathways (1), along with high total choline-containing compounds and phosphocholine levels (2). Using metabolomic profiling, tissue levels of sarcosine — an N-methyl derivative of the amino acid glycine and a metabolic product of choline (Fig. 1) — have been found to be elevated in localized prostate cancer compared to benign prostatic tissues. Tissue sarcosine levels have also been shown to increase during prostate cancer progression to metastatic disease (3), and knockdown of glycine-N-methyl transferase — the enzyme that generates sarcosine from glycine — attenuates prostate cancer invasion. Addition of exogenous sarcosine, or knockdown of the mitochondrial enzyme that leads to sarcosine degradation (sarcosine dehydrogenase [SARDH]) induces an invasive phenotype in benign prostate epithelial cells (4). Furthermore, there is evidence for a strong sarcosine-related induction of genes involved particularly in cell cycle progression (5). Sarcosine is therefore thought to be a potential onco-metabolite rather than a non-proteinogenic amino acid (6).

These data suggest a potential role of radiolabeled sarcosine for positron emission tomography (PET) imaging. <sup>11</sup>C-Choline and <sup>18</sup>F-fluorocholine are widely used PET radiopharmaceuticals for imaging of prostate cancer, particularly in the setting of prostate cancer recurrence (7). At primary staging, <sup>11</sup>C-choline preferentially identifies intermediate- and high-risk (i.e., clinically significant) prostate cancer, while low-risk lesions are typically not visualized (8,9). However, choline radiotracers are limited due to commonly observed increased

retention in nodular benign prostatic hyperplasia (10) as well as unsatisfactory accuracy for nodal metastatic disease in initial staging (11).

The purpose of this study was to assess the uptake mechanism of  $^{11}\text{C}$ -sarcosine, test its utility in common prostate cancer xenograft models in comparison to  $^{11}\text{C}$ -choline using small-animal PET (microPET), and to provide first human evidence of its potential in localized prostate cancer.

## **METHODS**

### Radiotracer Synthesis

$^{11}\text{C}$ -Choline was synthesized by  $^{11}\text{C}$ -methylation of N,N-dimethylaminoethanol with  $^{11}\text{C}$ -methyl iodide (12), while  $^{11}\text{C}$ -acetate was prepared by bubbling  $^{11}\text{C}$ - $\text{CO}_2$  through a solution of methyl-magnesium chloride (13).

We radiolabeled  $^{11}\text{C}$ -sarcosine by our standard  $^{11}\text{C}$ -methylation method using  $^{11}\text{C}$ -methyl triflate (14), followed by saponification using aqueous sodium hydroxide at 60 °C (Fig. 2). After labeling, the reaction mixture was diluted with water and purified using anion-exchange solid-phase extraction cartridge. The final injectable dose was eluted with aqueous sodium-chloride and filtered through a 0.22  $\mu\text{m}$  sterile filter. Total synthesis time was approximately 25 minutes. Non-decay corrected radiochemical yield was 2.5 - 6 % (non-decay-corrected yield based on 3 Ci of  $^{11}\text{C}$  of  $^{11}\text{C}$ CO<sub>2</sub>, n=5) and radiochemical purity > 95%. Radiochemical identity was confirmed and quantified by standard high-performance liquid-chromatography (i.e., calculating relative retention times). The mass of  $^{11}\text{C}$ -sarcosine for human use was  $\leq 10$  nmol.

## Cell culture and Animal Experiments

All animal experiments were conducted in accordance with the “Guide for the care and use of laboratory animals” (8th edition, National Research Council, National Academies Press, 2011), as well as with the institutional commission on the ethical treatment of animals.

Androgen receptor positive (LNCaP) and negative (DU-145 and PC-3) prostate cancer cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Genetically fingerprinted androgen receptor positive (LNCaP) and negative (DU-145, PC-3) prostate cancer cell lines were cultured in RPMI-1640 medium in the presence of 10 % heat-inactivated fetal bovine serum, 1 % Penicillin/Streptomycin, 2 mMol L-glutamine, and 1 mMol sodium pyruvate. Approximately 5 million LNCaP, DU-145 or PC-3 cells were injected into the flanks of 6 to 8 week-old athymic nu/nu mice (Charles River, Wilmington, MA). Animals were housed under pathogen-free conditions in micro-isolator cages, with rodent chow and water available ad libitum. Animals were examined daily and body weight and tumor size were determined. The tumors were allowed to grow to a diameter of 4 - 6 mm (short axis) before imaging.

## <sup>11</sup>C-Sarcosine Cell Assays

The <sup>11</sup>C-sarcosine uptake of approximately  $3 \times 10^5$  PC-3 and LNCaP tumor cells was determined (in triplicates) with and without presence of increasing concentrations of unlabeled sarcosine. To determine the precision of uptake measurements, PC-3 cells were incubated in RPMI-1640 medium for 20 minutes with 9 kBq of <sup>11</sup>C-sarcosine, washed 3-times with ice-cold

phosphate-buffered saline, and counted. The  $^{11}\text{C}$ -sarcosine uptake was determined to be 12.5 ( $\pm 1.1$ ) % of the administered dose ( $n = 12$ ).  $^{11}\text{C}$ -Sarcosine uptake was then determined using specific competitive inhibitors of two amino acid transporter systems, HT for the proton-coupled amino acid transporters (PAT1 gene symbol SLC36A1, PAT2 gene symbol SLC36A2, PAT4 gene symbol SLC36A4) (15), and BCH for system L (16). Thereafter,  $^{11}\text{C}$ -sarcosine was added to cells for 20 minutes and washed 3 times before counting. Using trypan blue staining, cell vitality was found intact ( $> 95\%$ ) after incubation and washing for all samples.

#### $^{11}\text{C}$ -Sarcosine Biodistribution

The biodistribution and organ kinetics of  $^{11}\text{C}$ -sarcosine were measured in normal rats and used to estimate human radiation absorbed doses according to previously described methods (17). Briefly, the organ distribution of  $^{11}\text{C}$ -sarcosine was determined in two male and two female Sprague-Dawley rats at five time points (5, 15, 30, 60 and 90 minutes) using injected doses of 7.4 – 28 MBq of  $^{11}\text{C}$ -sarcosine. Groups of animals were sacrificed while under isoflurane anesthesia. Organs were quickly removed, weighed and sectioned for gamma counting. The kinetics of elimination of  $^{11}\text{C}$ -sarcosine through urinary excretion were measured with dynamic PET imaging of the bladder in 4 rats (microPET P4 scanner, Siemens). A one-phase exponential association model was fitted to urinary bladder time-activity data to determine the fraction of the injected dose excreted into urine. The OLINDA/EXM 1.0 software package was used to generate human radiation absorbed dose estimates from the rat biodistribution kinetic data and the urinary excretion data, applying a 4.0 h bladder voiding interval (18).



## Radiolabeled Sarcosine Metabolites

High-performance liquid-chromatography was performed to assess for radiolabeled  $^{11}\text{C}$ -sarcosine metabolites in blood, and tissue homogenates of rat prostate and pancreas. Based on the known tissue metabolism of sarcosine as a methyl donor, a certain fraction of  $^{11}\text{C}$ -sarcosine is expected to be metabolized to  $^{11}\text{C}$ - $\text{CO}_2$  and exhaled (19). We measured the cumulative exhaled radioactivity for  $^{11}\text{C}$ -sarcosine ( $n = 2$ ), in comparison to  $^{11}\text{C}$ -acetate ( $n = 1$ ) and  $^{11}\text{C}$ -choline ( $n = 1$ ) as negative control. Using a closed system, the radioactivity in breathing air was captured with a commercial activated charcoal filter (EnviroPure, Travelers Rest, SC) and placed into the microPET scanner for 60 minutes to measure cumulative time-activity curves of exhaled air.

We also analyzed volatile radioactivity in tissues, assuming them to be  $^{11}\text{C}$ - $\text{CO}_2$ . Blood, pancreas, and prostate were collected and split into two pre-weighted vials. One was sealed immediately, while the other was treated with  $\text{HClO}_4$  (0.8 N) and homogenized. Nitrogen gas was then passed through the mixture for 2 minutes at about 10 mL/min flow rate to evaporate the volatile metabolite. The percentage of volatile metabolite from each sample was calculated based on activity loss and tissue weight.

## Target Metabolite Analyses

Absolute quantification of choline, glycine, and sarcosine in tumor tissue was determined by target metabolite validation using gas-chromatography mass-spectrometry-based assays in human prostate cancer tissues and compared to normal prostate tissues

collected by the institutional prostate tissue core. Briefly, frozen tissues were homogenized in methanol after spiking with labeled internal standards ( $d^3$ -sarcosine and  $^{13}C$ -glycine) and extracted using 1:1 volume ratio of water/chloroform. The aqueous methanolic layer was collected, dried and the extract was azeotroped twice with dimethylformamide.

Dimethylformamide (100  $\mu$ L) and *N*-methyl-*N*-*tert*-butylmethylsilyltrifluoroacetamide + 1 % *t*-butyl-dimethylchlorosilane were added and incubated at 60 °C for 1 hour. Selective ion monitoring was used for quantification. The amount of sarcosine and glycine was quantified by measuring the peak area of the native sarcosine ( $m/z = 232$ ) to that corresponding to spiked isotope-labeled sarcosine ( $m/z = 235$ ) and the native glycine ( $m/z = 218$ ) to that of spiked labeled glycine ( $m/z = 219$ ) respectively. The levels of sarcosine and glycine were normalized to the tissue weight (in nmol/mg).

Choline was extracted from tissues by liquid-liquid extraction (MeOH:H<sub>2</sub>O:CHCl<sub>3</sub>, 1:1:1 ratio). Tissues were homogenized after spiking the internal standard ( $^{13}C$ ,  $d^4$ - choline chloride). The aqueous methanolic layer containing choline was evaporated to dryness. After cooling, 50  $\mu$ L of pyridine and 100  $\mu$ L of *N,O*-Bis(trimethylsilyl)trifluoroacetamide were added and heated at 70°C for 60 min. The reaction mixture was dissolved in ethyl-acetate and injected for gas-chromatography mass-spectrometry. The amount of choline was calculated by measuring the peak area of the native choline ( $m/z = 146$ ) to that corresponding to spiked isotope-labeled choline ( $m/z = 151$ ).

$^{11}C$ -Sarcosine and  $^{11}C$ -choline MicroPET

Nude mice bearing DU-145 (n = 10) and PC-3 (n = 7) tumors received  $^{11}\text{C}$ -sarcosine ( $27.2 \pm 13$  KBq) intravenously through a bolus tail vein injection and a 30-minute dynamic microPET scan was performed. After near complete decay to  $< 2\%$  of initial dose, a dynamic  $^{11}\text{C}$ -choline ( $28 \pm 9$  KBq) scan was performed in the same position to ascertain identical tumor position between scans and to avoid bias due to rapidly growing tumors.

#### Data Reconstruction and Analysis

Images were corrected for decay, scatter and photon-attenuation using a Germanium-68 rod source. Time-activity curves of volumes of interest were obtained from dynamic image data sets defined for major organs and tumors to calculate tumor to muscle background ratios (TBR). The inspection of time-activity data indicated that the TBRs of  $^{11}\text{C}$ -sarcosine and  $^{11}\text{C}$ -choline were relatively stable between 5 - 20 minutes. We therefore selected this time interval for the comparison of the TBRs of both tracers.

#### First Human Subject

After internal review board approval and obtaining written consent, a patient with Gleason 4+3 primary prostate cancer received 606 MBq of  $^{11}\text{C}$ -sarcosine for PET/CT scanning (Siemens Biograph 40 TrueV mCT). Anatomic 3T magnetic resonance imaging (MRI) was performed separately (Philips Ingenia). PET data were registered with the 3D T2-weighted MRI using commercial software (MIM Maestro Software, Cleveland, OH) (20,21). The 3D T2-weighted sequence was then used for targeted transrectal prostate biopsies using a commercial system (UroNav, Invivo, Gainesville, FL).

## Statistics

Statistical analyses were performed using JMP 12 (SAS, Cary, NC). For paired data ( $^{11}\text{C}$ -sarcosine vs.  $^{11}\text{C}$ -choline), a paired Wilcoxon test was performed. For tissue metabolites, ANOVA followed by Student-Newman-Keuls tests were performed. Data are presented as mean  $\pm$  standard deviation, when appropriate, and *P*-values less than 0.05 were considered statistically significant.

## RESULTS

### MicroPET

Both,  $^{11}\text{C}$ -sarcosine and  $^{11}\text{C}$ -choline showed intense radiotracer uptake in the intestine and kidneys (Table 1). Similar to  $^{11}\text{C}$ -choline, the urinary excretion of radioactivity from  $^{11}\text{C}$ -sarcosine was limited.  $^{11}\text{C}$ -Sarcosine liver uptake was elevated compared to the mediastinum, but was markedly lower than the hepatic  $^{11}\text{C}$ -choline uptake. Low  $^{11}\text{C}$ -choline uptake was noted in the brain, but  $^{11}\text{C}$ -sarcosine uptake in brain was nearly absent (Fig. 3).

Time-activity curves of  $^{11}\text{C}$ -sarcosine showed rapid tumor uptake in both tumor models with relatively stable TBR after 5-minutes post injection. All 17 DU-145 and PC-3 tumors were visually identified on  $^{11}\text{C}$ -sarcosine PET, but only 12 of 17 (71 %) were noted with  $^{11}\text{C}$ -choline. The TBR (at 5-20 minutes) for  $^{11}\text{C}$ -sarcosine was significantly elevated compared to  $^{11}\text{C}$ -choline in both tumors models (Fig. 3). The  $^{11}\text{C}$ -sarcosine TBR of DU-145 tumors was determined as 1.92

$\pm 0.11$  compared to  $1.41 \pm 0.13$  for  $^{11}\text{C}$ -choline ( $p < 0.002$ ). Similarly, the TBR of PC-3 tumors was found to be  $1.89 \pm 0.2$  for  $^{11}\text{C}$ -sarcosine and  $1.34 \pm 0.16$  for  $^{11}\text{C}$ -choline ( $p < 0.002$ ).

## Cell Assays

The  $^{11}\text{C}$ -sarcosine uptake of PC-3 and LNCaP cells could be blocked with excess of non-radiolabeled (“cold”) sarcosine (LNCaP: decrease to  $3.2 \pm 0.7$  % at 1.125 mMol/mL; PC-3: decrease to  $1.1 \pm 0.4$  % at 7.5 mMol/mL), confirming a specific transport mechanism of sarcosine into cells. The PAT inhibitor HT and the system L inhibitor BCH were tested at 0.015 mMol/mL concentrations. After HT incubation,  $^{11}\text{C}$ -sarcosine uptake of PC-3 cells decreased to  $22.7 \pm 7.6$  % in a dose dependent fashion.  $^{11}\text{C}$ -Sarcosine uptake of LNCaP cells could be almost completely blocked by HT to  $9.6 (\pm 4.3)$  % compared to controls, confirming PAT-dependent transport of  $^{11}\text{C}$ -sarcosine. Since sarcosine is not an L-amino acid, BCH had little effect on  $^{11}\text{C}$ -sarcosine uptake of both cell lines (reduction to  $87.9 \pm 33.6$  % for PC-3, and to  $85.0 \pm 13.0$  % for LNCaP).

## Radiation Dosimetry Estimates

The biodistribution of  $^{11}\text{C}$ -sarcosine obtained from rats was used to calculate human radiation absorbed dose estimates (Table 1). The maximum percent injected dose observed in the gastrointestinal tract (9.0 %) was assumed to enter the small intestine. Also, based on separate rat microPET studies,  $2.6 \pm 1.4$  % of the injected dose were excreted via the urinary bladder with a biological half-time of  $0.152 \pm 0.081$  hours. Absorbed dose estimates are shown for the reference adult male organ model in Table 2. The organs with the highest absorbed

dose estimates were the small intestine followed by the kidneys. The 'effective dose' was calculated as 0.0045 mSv/MBq, resulting in 2.68 mSv for human subjects (at 600 MBq administered dose).

### Radiolabeled Metabolite Analyses

No aqueous radiolabeled metabolites of  $^{11}\text{C}$ -sarcosine in blood, prostate, or pancreas were detected at any time point. We concluded that the radiolabeled methyl-group of  $^{11}\text{C}$ -sarcosine is eliminated from the aqueous pool because of SARDH-mediated conversion to glycine (Fig. 1). This was further substantiated by capturing radioactivity in the exhaled air, indicating production of  $^{11}\text{C}$ - $\text{CO}_2$ . Cumulative time-activity curves obtained from expiratory air indicated that approximately 7 % of the injected dose was exhaled as  $^{11}\text{C}$ - $\text{CO}_2$  over a period of 60 minutes, which was markedly less compared to  $^{11}\text{C}$ -acetate, another known methyl-donor. In the case of  $^{11}\text{C}$ -acetate, the filter was unable to capture all exhaled  $^{11}\text{C}$ - $\text{CO}_2$  from  $^{11}\text{C}$ -acetate (Fig. 3 E). The cumulative time-activity curve obtained after  $^{11}\text{C}$ -sarcosine administration was flat from about 30 to 60 minutes, indicating that practically all  $^{11}\text{C}$ - $\text{CO}_2$  was exhaled within 30 minutes. The amount of radioactivity detected in the expired air after  $^{11}\text{C}$ -choline administration (as negative control) was negligible.

We further determined the volatile  $^{11}\text{C}$ - $\text{CO}_2$  fraction of radioactivity within blood, pancreas, and prostate in one animal per time point (15, 30 and 60-minutes post administration). The  $^{11}\text{C}$ - $\text{CO}_2$  fraction ranged from 14 – 21 % in blood, 9 – 23 % in prostate, and

12 – 24 % in pancreas. Thus, a substantial majority of radioactivity was related to  $^{11}\text{C}$ -sarcosine and not  $^{11}\text{C}$ - $\text{CO}_2$  in every measured tissue at all time points.

### Human Subject

Fig. 4 shows hybrid PET/CT images of the first human subject injected with  $^{11}\text{C}$ -sarcosine. Early time-point images (between 5 - 10 minutes post injection) displayed focally increased uptake in the right anterior transition zone of the prostate gland and no significant uptake within contralateral benign prostatic hyperplasia nodules. Registration of PET with T2-weighted MR images revealed a low-signal intensity lesion at this location, measuring 1.04 mL on T2-weighted MR. Using image-guided prostate biopsy, a Gleason 4+3 prostate cancer was identified, while standard biopsies were negative. Time-activity curves demonstrated preferential  $^{11}\text{C}$ -sarcosine uptake in the tumor compared to the total prostate and the arterial blood with stable lesion-to-background ratio over time (Fig. 5).

### Metabolite Analyses

Human prostate tissue concentrations of choline (n = 6) increased significantly from benign ( $0.17 \pm 0.05$ ), to localized ( $0.28 \pm 0.08$ ) and metastatic ( $0.42 \pm 0.08$  nmol/mg) prostate cancer (statistical significance levels as listed in Fig. 6). Similarly, glycine (n = 10) showed significant stepwise increases from benign prostate tissues ( $1.21 \pm 0.18$ ) to localized ( $1.67 \pm 0.41$ ) to metastatic ( $4.07 \pm 1.53$  nmol/mg) prostate cancer, with sarcosine (n = 10) displaying

the largest gains (benign:  $1.54 \pm 0.6$ ; localized:  $3.82 \pm 2.08$ ; metastatic prostate cancer:  $15.57 \pm 8.0$  pmol/mg).

## DISCUSSION

We identified increased  $^{11}\text{C}$ -sarcosine uptake (relative to background) in two preclinical human prostate cancer xenograft models, with moderate improvement over  $^{11}\text{C}$ -choline. Furthermore, initial human data are promising in a case of low-volume organ-confined prostate cancer, further substantiating a utility for human use. We considered  $^{11}\text{C}$ -sarcosine for prostate cancer imaging since metabolomic data indicated strongly elevated tissue levels in localized prostate cancer, particularly in metastatic disease (3). Also, the mere addition of sarcosine to cultured benign prostate epithelial cells induced an invasive phenotype (4). While the sarcosine metabolism is well-characterized, its metabolic functions in the human body are still poorly understood and are currently under active investigation (5,22).

Radiolabeled  $^{11}\text{C}$ -sarcosine is chemically identical to the non-proteinogenic amino acid sarcosine. The known metabolism of (unlabeled) sarcosine consists of two biochemical pathways occurring in mitochondria. The first pathway is the oxidative pathway of choline via betaine and dimethylglycine (Fig. 1). Here, the enzyme SARDH plays a critical role in the conversion to glycine. The second pathway involves methionine with donation of a methyl-group via S-adenosylmethionine to glycine. Here, glycine N-methyltransferase acts as an essential component that influences synthesis of sarcosine from glycine (23). Since  $^{11}\text{C}$ -



sarcosine is not a substrate in either pathway, we consider  $^{11}\text{C}$ -sarcosine cell uptake not to reflect the activity of these sarcosine production pathways.

Sarcosine is a known ligand of the PAT family, which are thought as multi-purpose carriers with distinct roles in different cells (24). PAT1/PAT2 are low affinity carriers that mediate 1:1 symport of protons and small amino acids such as glycine, alanine, proline (25), as well as gamma-aminobutyric acid into neurons. PAT4 is a high-affinity, low capacity electroneutral transporter of neutral amino acids (26). Baseline PAT expression is ubiquitous (15) and resides at the cell surface and intracellular membranes (endosomes, lysosomes) (27). Elevated PAT expressions have been linked to cell proliferation (24). Here, PATs function as part of an amino acid-sensing engine that drives activation of the mammalian target of rapamycin complex 1 (mTORC1) (27). Physiologically high PAT1/ PAT2 expressions have been identified in brain, pancreas, kidney, duodenum, and small intestine (24). SLC36A2 expression has been documented at the apical surface of the human proximal renal tubule, where PAT2 functions in the reabsorption of amino acids (24). Within the intestinal tract, PAT1 plays an important role as nutrient and drug transporter (28). PAT4 (SLC36A4) is abundantly expressed in excitatory and inhibitory neurons as well as epithelial cells (26). In cancer, PAT4 is particularly important for the amino acid-dependent activation of the mTORC1 pathway, which is upregulated in prostate and colon cancer, where PAT4 overexpression has been linked to an aggressive phenotype (29).

In prostate cancer cells,  $^{11}\text{C}$ -sarcosine uptake could be blocked with “cold” sarcosine, which confirmed a specific transport mechanism for sarcosine and suggests transport as the

rate-limiting step. The dose-dependent inhibition of  $^{11}\text{C}$ -sarcosine uptake by the PAT inhibitor HT and the lack of significant inhibition by BCH are consistent with the literature surrounding sarcosine. While we have not verified PAT expression in the tested PC-3 and LNCaP cancer cell lines, we nevertheless conclude that transport of  $^{11}\text{C}$ -sarcosine into cells is PAT-mediated.

The  $^{11}\text{C}$ -sarcosine biodistribution in normal rats indicated elevated uptake in the pancreas and kidneys, moderate uptake in the liver, stomach, lungs, and adrenals, while all other major parenchymal organs displayed low uptake. The elevated  $^{11}\text{C}$ -sarcosine uptake in pancreas and kidneys is likely related to PAT1/PAT2 regulated transport. However,  $^{11}\text{C}$ -sarcosine uptake in rat brain was found to be low despite neuronal PAT1 expression. Also, organs with elevated SARDH protein expression (high protein levels are found in the liver, pancreas and adrenal glands (30)) might also have elevated  $^{11}\text{C}$ -sarcosine uptake, which – based on our biodistribution data – appears to be the case.

Radiation dosimetry estimates for  $^{11}\text{C}$ -sarcosine resulted in an ‘effective dose’ of 0.0045 mSv/MBq, which is very similar to that of  $^{11}\text{C}$ -choline and compares favorably to  $^{18}\text{F}$ -FDG and many other Carbon-11 labeled radiopharmaceuticals (31). Our absorbed radiation dose measurements confirmed that  $^{11}\text{C}$ -sarcosine can be safely administered to humans.

$^{11}\text{C}$ -Choline and  $^{18}\text{F}$ -fluorocholine are widely used PET radiotracers for prostate cancer imaging (9,20), but also  $^{11}\text{C}$ -methionine and  $^{11}\text{C}$ -acetate PET have been successfully tested for this indication (32,33).  $^{11}\text{C}$ -Choline is known to be rapidly transported into human cancers,

including prostate cancer. While the uptake of  $^{11}\text{C}$ -choline has been linked to the choline kinase activity in prostate cancer (34), a substantial fraction of  $^{11}\text{C}$ -choline may remain non-metabolized intracellularly (35), indicating that choline transport and not phosphorylation may be key to  $^{11}\text{C}$ -choline uptake into certain cancer cells. In this context, one potential basis for elevated sarcosine tissue levels would be the upregulation of the oxidative pathway of choline to glycine. In fact using  $^{11}\text{C}$ -choline, it was determined that oxidation of choline is the dominant metabolic pathway in HCT116 tumor xenografts (36). Our data obtained from human tissues indicate significantly increased choline, sarcosine and glycine tissue levels in prostate cancer and particularly in metastatic disease compared to benign prostatic tissues. These data are compatible with an upregulation of both metabolic pathways leading to sarcosine production: a) the methylation from glycine via glycine-N-methyl transferase, as well as b) the oxidative pathway of choline. While activation of these pathways will increase the sarcosine tissue concentration, we consider the  $^{11}\text{C}$ -sarcosine uptake only to reflect PAT transport activity and not the magnitude of the sarcosine tissue pool.

Our metabolite analyses did not reveal any radiolabeled aqueous metabolites in blood and tissue. We attribute this finding to the fact that with the demethylation of  $^{11}\text{C}$ -sarcosine to glycine by SARDH, the radiolabeled carbon group is ultimately converted to  $^{11}\text{C}$ - $\text{CO}_2$ . Therefore, the measured tissue radioactivity will be determined largely by the sum of (unchanged)  $^{11}\text{C}$ -sarcosine and  $^{11}\text{C}$ - $\text{CO}_2$ . Since  $^{11}\text{C}$ - $\text{CO}_2$  will gradually be removed from tissues, it is unclear to whether the decrease of radioactivity from tissue over time (as identified by biodistribution studies) is related to  $^{11}\text{C}$ -sarcosine and/or  $^{11}\text{C}$ - $\text{CO}_2$ . However, we do not believe that the decline

in the radioactivity noted in many organs is related to a significant loss of  $^{11}\text{C-CO}_2$  (Table 1). Such declines are commonly seen for many Carbon-11 labeled radiopharmaceuticals without  $^{11}\text{C-CO}_2$  production, including  $^{11}\text{C-choline}$  as unbound radioactivity is escaping back into the vasculature. Since tissues were quickly excised and placed into air-tight vials for biodistribution, a potential for a loss of radioactivity due to  $^{11}\text{C-CO}_2$  evaporation was minimized. In fact, the lack of such decline from tumor tissue, in contrast to benign prostate tissues as seen in Fig. 5, may not only indicate prostate cancer, but may have adverse prognostic implications. First, elevated sarcosine tissue levels and enzymes related to the sarcosine metabolism have been linked to tumor aggressiveness not only in prostate cancer (3,4), but also in breast and hepatocellular carcinomas where increased GNMT protein expression has been associated with decreased survival (37,38). Thus, we postulate that  $^{11}\text{C-sarcosine}$  kinetics (displaying a loss of radioactivity from tumor tissue over time), whether due to demethylation of  $^{11}\text{C-sarcosine}$  by SARDH resulting in  $^{11}\text{C-CO}_2$  production or due to an equilibrium of  $^{11}\text{C-sarcosine}$  with (low) intracellular non-labeled sarcosine levels, might indicate less aggressive tumor features and may have prognostic value.

## **Conclusions**

Despite significant progress, the understanding of PAT transporters and their signaling functions is far from complete. The importance of the activity of SLC36 family amino acid (PAT) transporters in cell proliferation and cancer has only recently been fully recognized (22). Here, our data suggest that PAT-mediated sarcosine transport is key to  $^{11}\text{C-sarcosine}$  uptake in

prostate cancer.  $^{11}\text{C}$ -Sarcosine PET allows the assessment of PAT transport in the human body with apparent utility in cancer imaging. The improved tumor visualization with  $^{11}\text{C}$ -sarcosine compared to  $^{11}\text{C}$ -choline seen in xenograft tumor models together with increased sarcosine tumor tissue levels compared to benign prostate tissues indicate a potential role for  $^{11}\text{C}$ -sarcosine PET in human prostate cancer. If proven successful with further human use, this radiotracer exemplifies a new concept for PET imaging of cancer based on proton-coupled SLC36 family amino acid transporters currently undergoing active research.

#### **ACKNOWLEDGMENTS**

We thank the staff of the clinical and preclinical PET suites for their excellent technical support. Funded by NIH R21CA191052-01, NIH DK097153, and a seed grant of the Radiology Department of the University of Michigan.

## LITERATURE

1. Burton AJ, Tilling KM, Holly JM, et al. Metabolic imbalance and prostate cancer progression. *Int J Mol Epidemiol Genet.* 2010;1:248-271.
2. Swanson MG, Keshari KR, Tabatabai ZL, et al. Quantification of choline- and ethanolamine-containing metabolites in human prostate tissues using 1H HR-MAS total correlation spectroscopy. *Magn Reson Med.* 2008;60:33-40.
3. Sreekumar A, Poisson LM, Rajendiran TM, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature.* 2009;457:910-914.
4. Khan AP, Rajendiran TM, Ateeq B, et al. The role of sarcosine metabolism in prostate cancer progression. *Neoplasia.* 2013;15:491-501.
5. Heger Z, Merlos Rodrigo MA, Michalek P, et al. Sarcosine Up-Regulates Expression of Genes Involved in Cell Cycle Progression of Metastatic Models of Prostate Cancer. *PLoS One.* 2016;11:e0165830.
6. Cha YJ, Kim do H, Jung WH, Koo JS. Expression of sarcosine metabolism-related proteins according to metastatic site in breast cancer. *Int J Clin Exp Pathol.* 2014;7:7824-7833.
7. Picchio M, Briganti A, Fanti S, et al. The role of choline positron emission tomography/computed tomography in the management of patients with prostate-specific antigen progression after radical treatment of prostate cancer. *Eur Urol.* 2011;59:51-60.
8. Park H, Wood D, Hussain H, et al. Introducing parametric fusion PET/MRI of primary prostate cancer. *J Nucl Med.* 2012;53:546-551.
9. Piert M, Park H, Khan A, et al. Detection of aggressive primary prostate cancer with 11C-choline PET/CT using multimodality fusion techniques. *J Nucl Med.* 2009;50:1585-1593.
10. Souvatzoglou M, Weirich G, Schwarzenboeck S, et al. The sensitivity of [11C]choline PET/CT to localize prostate cancer depends on the tumor configuration. *Clin Cancer Res.* 2011;17:3751-3759.
11. Piert M, El Naqa I, Davenport MS, Incerti E, Mapelli P, Picchio M. PET/MRI and prostate cancer. *Clin Transl Imaging.* 2016:1-13.

12. Shao X, Hockley B, Hoareau R, Schnau P, Scott PJH. High efficiency, fully automated preparation and quality control of [11C]choline and [18F]fluoromethylcholine for routine clinical application. *Appl Radiat Isot.* 2011;69:403-409.
13. Runkle AC, Shao X, Tluczek LJ, Henderson BD, Hockley BG, Scott PJ. Automated production of [11C]acetate and [11C]palmitate using a modified GE Tracerlab FX(C-Pro). *Appl Radiat Isot.* 2011;69:691-698.
14. Shao X, Kilbourn MR. A simple modification of GE tracerlab FX C Pro for rapid sequential preparation of [11C]carfentanil and [11C]raclopride. *Appl Radiat Isot.* 2009;67:602-605.
15. Alexander SP, Benson HE, Faccenda E, et al. The Concise Guide to PHARMACOLOGY 2013/14: transporters. *Br J Pharmacol.* 2013;170:1706-1796.
16. Kim CS, Cho SH, Chun HS, et al. BCH, an inhibitor of system L amino acid transporters, induces apoptosis in cancer cells. *Biological & pharmaceutical bulletin.* 2008;31:1096-1100.
17. Jang KS, Jung YW, Gu G, et al. 4-[18F]Fluoro-m-hydroxyphenethylguanidine: a radiopharmaceutical for quantifying regional cardiac sympathetic nerve density with positron emission tomography. *J Med Chem.* 2013;56:7312-7323.
18. Stabin MG, Sparks RB, Crowe E. OLINDA/EXM: the second-generation personal computer software for internal dose assessment in nuclear medicine. *J Nucl Med.* 2005;46:1023-1027.
19. Mitchell AD, Benevenga NJ. Importance of sarcosine formation in methionine methyl carbon oxidation in the rat. *J Nutr.* 1976;106:1702-1713.
20. Piert M, Montgomery J, Kunju LP, et al. 18F-choline PET/MRI: the Additional Value of PET for MRI-guided Transrectal Prostate Biopsies. *J Nucl Med.* 2016;57:1065-1070.
21. Meyer C, Ma B, Kunju LP, Davenport M, Piert M. Challenges in accurate registration of 3-D medical imaging and histopathology in primary prostate cancer. *Eur J Nucl Med Mol Imaging.* 2013;40 Suppl 1:72-78.
22. Zheng L, Zhang W, Zhou Y, Li F, Wei H, Peng J. Recent Advances in Understanding Amino Acid Sensing Mechanisms that Regulate mTORC1. *Int J Mol Sci.* 2016;17:1636-1651.
23. Cernei N, Heger Z, Gumulec J, et al. Sarcosine as a potential prostate cancer biomarker--a review. *Int J Mol Sci.* 2013;14:13893-13908.

24. Thwaites DT, Anderson CM. The SLC36 family of proton-coupled amino acid transporters and their potential role in drug transport. *Br J Pharmacol*. 2011;164:1802-1816.
25. Boll M, Daniel H, Gasnier B. The SLC36 family: proton-coupled transporters for the absorption of selected amino acids from extracellular and intracellular proteolysis. *Pflugers Arch*. 2004;447:776-779.
26. Roshanbin S, Hellsten SV, Tafreshiha A, Zhu Y, Raine A, Fredriksson R. PAT4 is abundantly expressed in excitatory and inhibitory neurons as well as epithelial cells. *Brain Res*. 2014;1557:12-25.
27. Ogmundsdottir MH, Heublein S, Kazi S, et al. Proton-assisted amino acid transporter PAT1 complexes with Rag GTPases and activates TORC1 on late endosomal and lysosomal membranes. *PLoS One*. 2012;7:e36616.
28. Anderson CM, Grenade DS, Boll M, et al. H+/amino acid transporter 1 (PAT1) is the imino acid carrier: An intestinal nutrient/drug transporter in human and rat. *Gastroenterology*. 2004;127:1410-1422.
29. Stevens D, Verrill C, Bryant R, et al. The proton-assisted amino acid transporter 4 (PAT4/SLC36A4) is up-regulated in prostate cancer. *The Journal of Urology*. 2015;193:e677.
30. Bergeron F, Otto A, Blache P, et al. Molecular cloning and tissue distribution of rat sarcosine dehydrogenase. *Eur J Biochem*. 1998;257:556-561.
31. Tolvanen T, Yli-Kerttula T, Ujula T, et al. Biodistribution and radiation dosimetry of [(11)C]choline: a comparison between rat and human data. *Eur J Nucl Med Mol Imaging*. 2010;37:874-883.
32. Toth G, Lengyel Z, Balkay L, Salah MA, Tron L, Toth C. Detection of prostate cancer with 11C-methionine positron emission tomography. *J Urol*. 2005;173:66-69.
33. Brogsitter C, Zophel K, Kotzerke J. 18F-Choline, 11C-choline and 11C-acetate PET/CT: comparative analysis for imaging prostate cancer patients. *Eur J Nucl Med Mol Imaging*. 2013;40 Suppl 1:S18-27.
34. Contractor K, Challapalli A, Barwick T, et al. Use of [11C]choline PET-CT as a noninvasive method for detecting pelvic lymph node status from prostate cancer and relationship with choline kinase expression. *Clin Cancer Res*. 2011;17:7673-7683.
35. Bansal A, Shuyan W, Hara T, Harris RA, Degrado TR. Biodisposition and metabolism of [(18)F]fluorocholine in 9L glioma cells and 9L glioma-bearing fisher rats. *Eur J Nucl Med Mol Imaging*. 2008;35:1192-1203.



- 36.** Witney TH, Alam IS, Turton DR, et al. Evaluation of deuterated 18F- and 11C-labeled choline analogs for cancer detection by positron emission tomography. *Clin Cancer Res.* 2012;18:1063-1072.
- 37.** Yoon JK, Kim do H, Koo JS. Implications of differences in expression of sarcosine metabolism-related proteins according to the molecular subtype of breast cancer. *J Transl Med.* 2014;12:149.
- 38.** Lim SO, Park SJ, Kim W, et al. Proteome analysis of hepatocellular carcinoma. *Biochem Biophys Res Commun.* 2002;291:1031-1037.

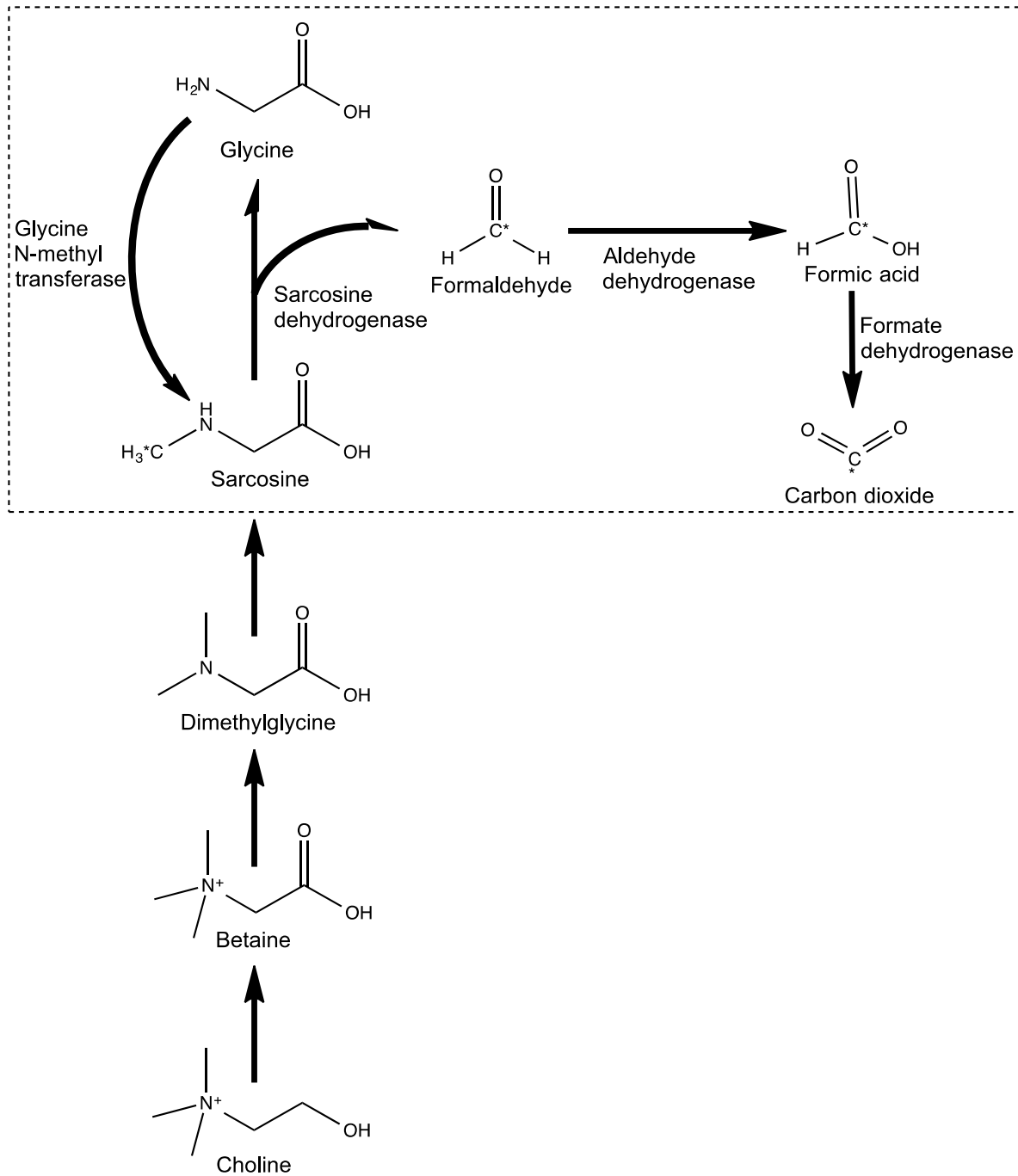


Fig. 1: Sarcosine Metabolism

C\* indicating radiolabel

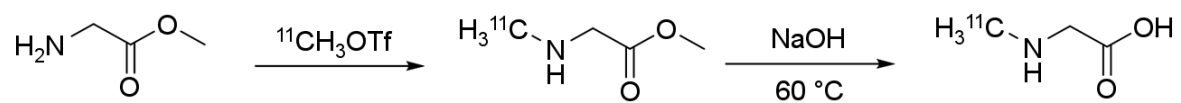


Fig. 2: Synthesis of N-<sup>11</sup>C-Methylglycine

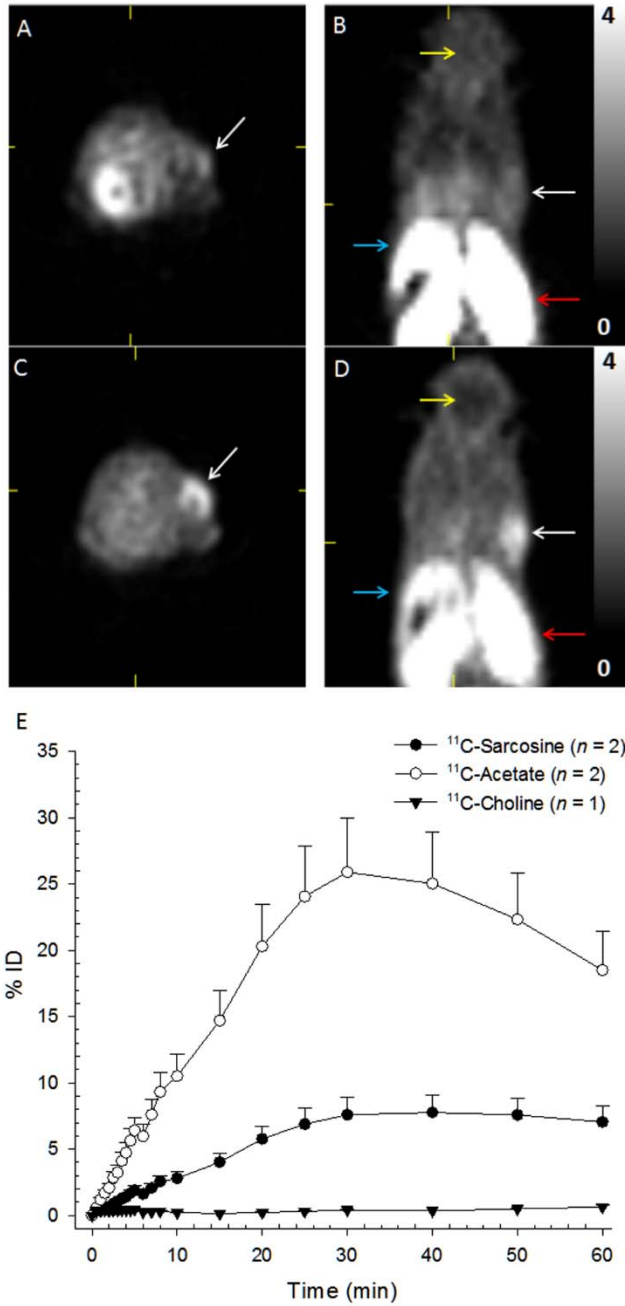


Fig. 3:  $^{11}\text{C}$ -Sarcosine and  $^{11}\text{C}$ -Choline microPET

Improved visualization of a DU-145 tumor (white arrows) and lower hepatic uptake (blue arrows) after intravenous injection of 16.3 MBq  $^{11}\text{C}$ -sarcosine (C, D) compared to 16.8 MBq of  $^{11}\text{C}$ -choline (A, C) on transaxial (A, C) and coronal (B, D) microPET images (summed data 5 – 20

min; SUV range 0 - 4). Both tracers display intense renal uptake (red arrows). Brain uptake (yellow arrows) is essentially absent for  $^{11}\text{C}$ -sarcosine, while  $^{11}\text{C}$ -choline brain uptake is low. The cumulative exhaled mean ( $\pm$  SD) radioactivity (as % injected dose) representing  $^{11}\text{C}$ - $\text{CO}_2$  (E), was measured in healthy rats after intravenous injection of  $^{11}\text{C}$ -sarcosine (black dots),  $^{11}\text{C}$ -acetate (white dots), and  $^{11}\text{C}$ -choline (black triangle).

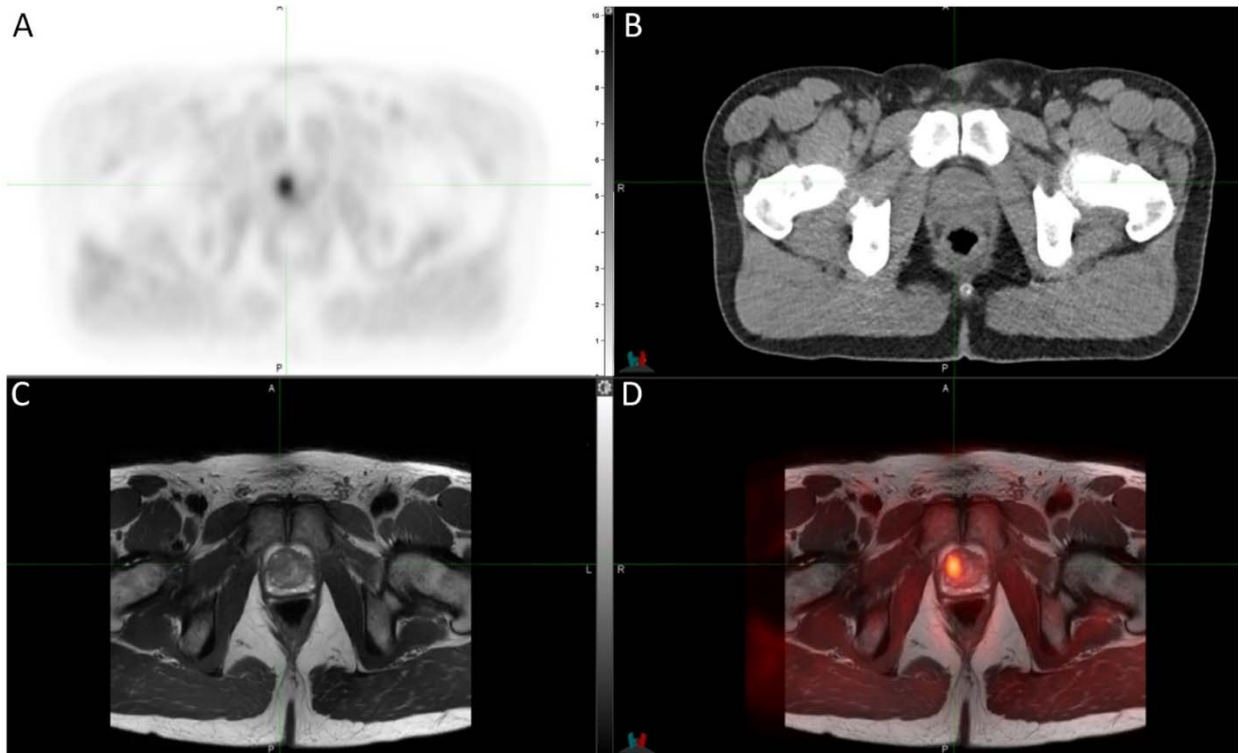


Fig. 4: Human  $^{11}\text{C}$ -sarcosine PET

Transaxial  $^{11}\text{C}$ -sarcosine hybrid PET/CT showed a (triangulated) adenocarcinoma in the transition zone of the anterior right prostate gland on PET (A), CT (B), and a separately obtained T2-weighted MR sequence (C) with resulting PET/MRI registration (D).

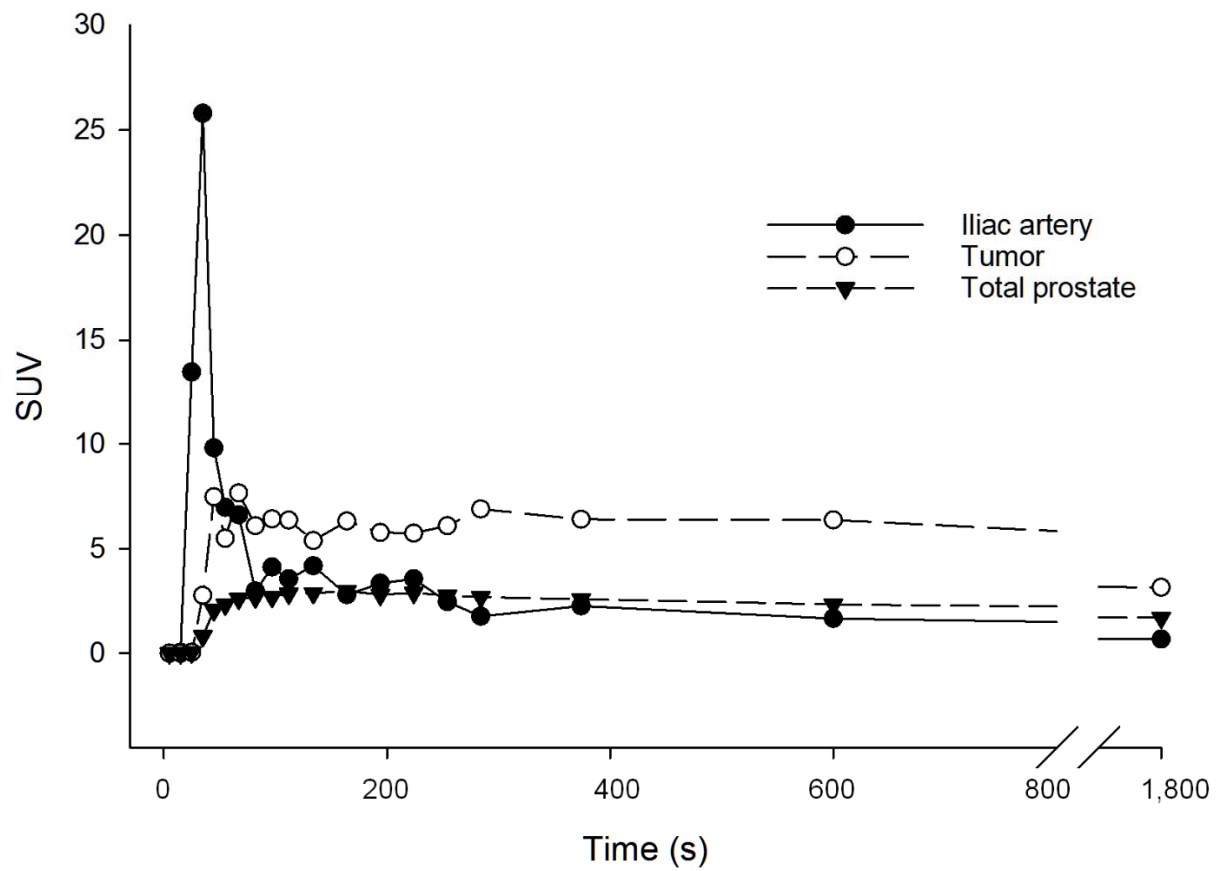


Fig. 5: Human  $^{11}\text{C}$ -Sarcosine Time-Activity Curves

Time-activity curves of the human subject indicated rapid tumor uptake of  $^{11}\text{C}$ -sarcosine (white dots) after delivery via the iliac artery (black dots).

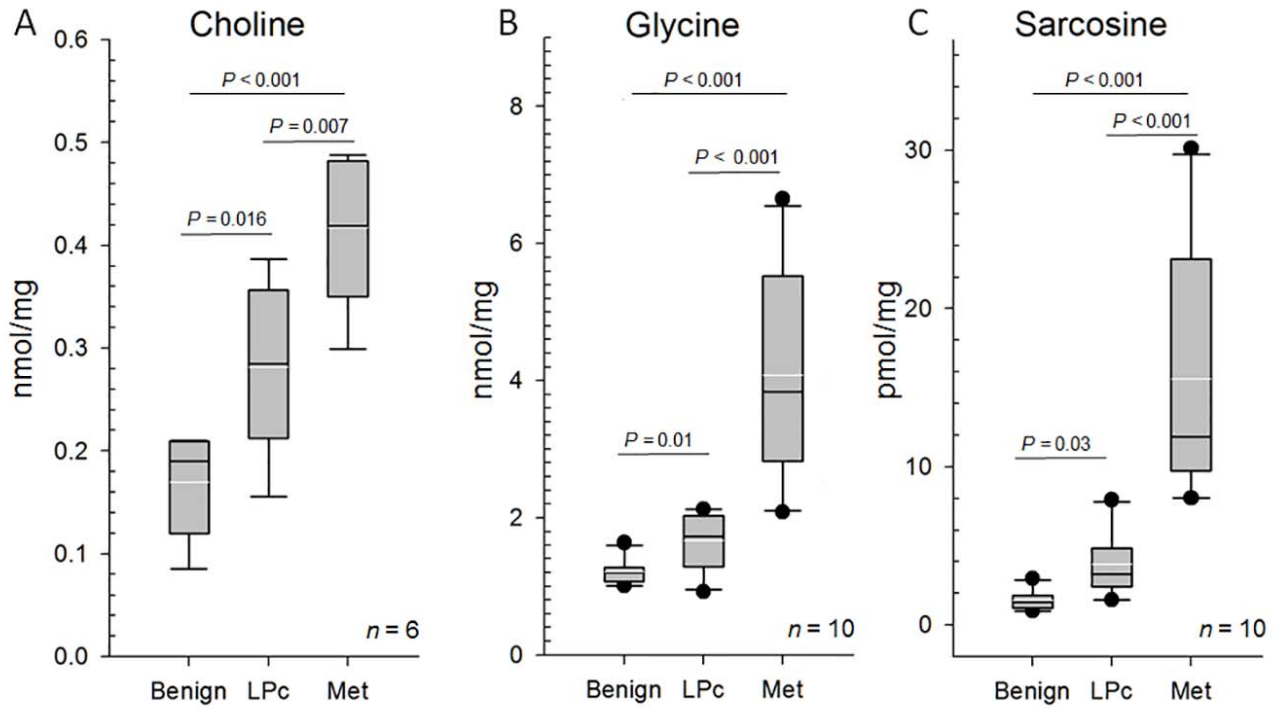


Fig. 6: Target Metabolite Analyses

Whisker plots (white lines: mean ( $\pm$  95 % confidence interval), black line: median, black dots: outliers) of tissue concentrations for choline (A), glycine (B) and sarcosine (C) obtained from human localized (LPc) and metastatic (Met) prostate cancer in comparison to benign prostate tissues (Benign).



Table 1

Biodistribution of  $^{11}\text{C}$ -sarcosine in major organs of rats (% ID/g).

Organ	5 min	15 min	30 min	60 min	90 min
Brain	0.090 ± 0.021	0.109 ± 0.014	0.138 ± 0.042	0.114 ± 0.031	0.115 ± 0.051
Eyeballs	0.194 ± 0.034	0.212 ± 0.028	0.168 ± 0.049	0.115 ± 0.030	0.111 ± 0.022
Heart	0.462 ± 0.130	0.455 ± 0.028	0.325 ± 0.040	0.217 ± 0.036	0.198 ± 0.049
Lung	1.049 ± 0.126	1.159 ± 0.179	0.821 ± 0.145	0.531 ± 0.056	0.467 ± 0.094
Liver	1.291 ± 0.541	1.100 ± 0.354	1.013 ± 0.417	0.633 ± 0.169	0.662 ± 0.159
Pancreas	1.068 ± 0.618	1.218 ± 0.370	1.106 ± 0.153	1.045 ± 0.695	0.960 ± 0.551
Spleen	0.626 ± 0.041	0.623 ± 0.125	0.493 ± 0.041	0.392 ± 0.011	0.398 ± 0.091
Adrenal	0.620 ± 0.166	0.555 ± 0.021	0.503 ± 0.056	0.398 ± 0.153	0.474 ± 0.318
Kidney	3.004 ± 1.114	2.776 ± 0.607	1.774 ± 0.362	1.260 ± 0.254	0.956 ± 0.129
Stomach	1.032 ± 0.319	1.033 ± 0.074	0.861 ± 0.169	0.610 ± 0.097	0.541 ± 0.050
Ovary*	0.863 ± 0.170	0.786 ± 0.031	0.727 ± 0.063	0.432 ± 0.004	0.375 ± 0.008
Uterus*	0.724 ± 0.068	0.508 ± 0.200	0.447 ± 0.169	0.522 ± 0.220	0.181 ± 0.018
Testes*	0.071 ± 0.054	0.172 ± 0.012	0.122 ± 0.007	0.114 ± 0.025	0.079 ± 0.001
Muscle	0.140 ± 0.040	0.206 ± 0.015	0.224 ± 0.047	0.226 ± 0.067	0.164 ± 0.032
Bone	0.752 ± 0.543	0.604 ± 0.071	0.506 ± 0.076	0.379 ± 0.038	0.265 ± 0.101
Blood	0.396 ± 0.247	0.423 ± 0.028	0.245 ± 0.054	0.179 ± 0.034	0.196 ± 0.035

\* ( $n = 2$ ) animals used for these values.

Table 2

Radiation absorbed dose estimates for  $^{11}\text{C}$ -sarcosine

Target organ	Total dose	
	(mGy/MBq)	(rad/mCi)
Adrenals	0.0040	0.015
Brain	0.0021	0.008
Breasts	0.0021	0.008
Gallbladder wall	0.0038	0.014
LLI wall	0.0034	0.013
Small intestine	0.0145	0.054
Stomach wall	0.0041	0.015
ULI wall	0.0066	0.025
Heart wall	0.0031	0.012
Kidneys	0.0115	0.042
Liver	0.0066	0.024
Lungs	0.0050	0.018
Muscle	0.0025	0.009
Ovaries	0.0065	0.024
Pancreas	0.0066	0.024
Red marrow	0.0024	0.009
Osteogenic cells	0.0035	0.013
Skin	0.0020	0.007
Spleen	0.0040	0.015
Testes	0.0023	0.009
Thymus	0.0025	0.009
Thyroid	0.0024	0.009
Urinary bladder wall	0.0086	0.032
Uterus	0.0057	0.021
Total body	0.0028	0.011
	(mSv/MBq)	(rem/mCi)
Effective dose	0.0045	0.017