Unique Protocol ID: IRB00061518

NCT01808222

Transmolecular Imaging of Recurrent Prostate Carcinoma with Exploration of Genomic Markers differences between Local and Distant Recurrence

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Date: 12/04/2014

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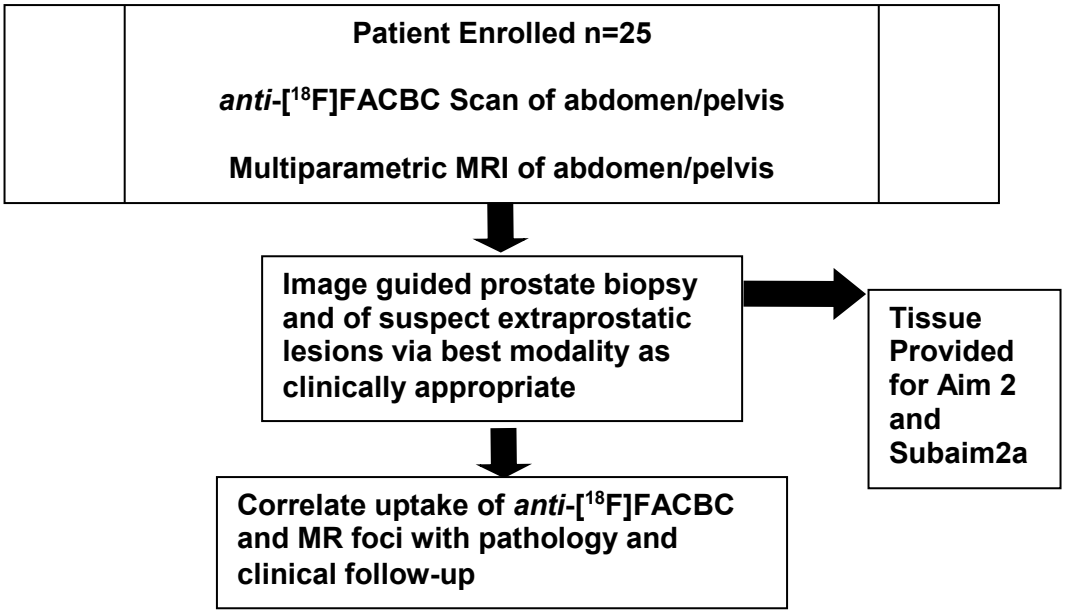
Schema


Patient Enrolled n=25
anti-[18F]FACBC Scan of abdomen/pelvis
Multiparametric MRI of abdomen/pelvis
Image guided prostate biopsy and of suspect extraprostatic lesions via best modality as clinically appropriate
Tissue Provided for Aim 2 and Subaim2a
Correlate uptake of *anti*-[18F]FACBC and MR foci with pathology and clinical follow-up

Precis/Abstract

Prostate cancer is the most common solid tumor, with approximately 200,000 new cases diagnosed per year. Several different local therapies are available for treatment, including surgery and radiotherapy [1]. Significant advances have been made in the technical aspects of surgery and of radiotherapy that have improved both the cancer control outcomes as well as the morbidity of treatment.

Despite these significant advances, approximately 30% of patients treated with definitive local therapy experience recurrent disease [2, 3]. **The differentiation of local from regional and from distant recurrence is of critical importance since salvage techniques can cure disease confined to the surgical field.** If pelvic nodal involvement is suspected, radiation fields can be extended to include the pelvic nodes [4-6]. If a patient is not a candidate for salvage radiotherapy, he will likely be treated with systemic long-term hormonal therapy that is expensive and can result in significant morbidity, also leading to increased healthcare expenditures.

In addition it has been noted in the literature that there may be genotypic and phenotypic differences between prostate carcinoma recurrence in the prostate bed and in extraprostatic nodal locations. Exome sequence analyses of prostate cancer metastases have identified mutations specific to castrate-resistant metastases including components of the Wnt pathway [7], while microarray studies have identified changes in gene expression specific to bone metastases including IGFBP2, WHSC2, and CRIM1 [8]. Integrative copy number and gene expression analyses have also identified important differences between primary and metastatic lesions including components of the AR pathways such as NCOA2 [9] and the polycomb complex chromatin suppressor EZH2 [10].

Imaging has the potential to play a central role in the detection of recurrent prostate carcinoma and in the differentiation of prostatic from extraprostatic recurrence. Yet to date, **no one method has demonstrated definitive accuracy in this regard.** For this reason, newer methods such as diffusion weighted MR (DWMR) and positron emission tomography (PET) with molecular radiotracers are currently under study for the characterization of post therapy recurrence [11-19]. Each of these techniques has its own strengths and weaknesses and each interrogates only a portion of the anatomic and imaging biomarker data present in recurrent prostate carcinoma.

The specific hypothesis in this proposal is that a combination of molecular interrogation with the synthetic amino acid PET radiotracer *anti*-[^{18}F]FACBC PET-CT combined with multiparametric MR including DWMR will provide a greater degree of imaging biomarker data than each technique individually. We believe that such transmolecular interrogation will enable us to more accurately diagnose recurrent prostate carcinoma within the prostate bed and in extraprostatic locations. We can then utilize tissue obtained from this study to examine the second specific hypothesis that there are genotypic differences between prostate carcinoma recurrence confined to the prostate bed and those that are extraprostatic, and also be useful to elucidate amino acid transport uptake mechanisms of *anti*-[^{18}F]FACBC.

A. Background and Significance

One in six men will develop prostate cancer [20]. Due to the widespread use of PSA testing, cancer is often detected before systemic disease occurs [2]. Depending on clinical presentation, therapy is typically approached through locally directed interventions such as radical prostatectomy, brachytherapy, external beam radiotherapy or cryotherapy. Yet, approximately, 30% of patients treated with definitive local therapy experience recurrent disease [2, 3].

Recurrent disease usually manifests with rising PSA. The differentiation of local from extraprostatic recurrence is of critical importance since salvage techniques can cure disease confined to the prostate bed. If pelvic nodal involvement is suspected, radiation fields can be extended to include the pelvic nodes [4-6]. Systemic disease is treated with hormonal manipulation and/or chemotherapy. PSA level is not useful in the differentiation of local from extraprostatic recurrence, though the rate of PSA rise may have some value [21].

Imaging plays a central role in the detection of recurrent prostate carcinoma in the prostate bed and in the differentiation of prostatic from extraprostatic recurrence. Conventional methodology including computed tomography (CT), magnetic resonance imaging (MR), transrectal ultrasound, bone scan and ^{111}Indium-capromab-pendetide (ProstaScint) (EUSA Pharma, Langhorne, PA) may provide important information, but suffer from less than optimal diagnostic performance [22-30]. Newer methods such as diffusion weighted MR (DWMR) and positron emission tomography (PET) with molecular radiotracers are currently under study for the characterization of post-therapy recurrence [11-19]. MR with superparamagnetic (USPIO) particles has shown promise but is not available in the US [19]. The sensitivity of most imaging techniques is dependent upon PSA level, doubling time, and velocity [16, 31, 32].

One PET radiotracer which has shown promise in the staging and restaging of patients with prostate carcinoma is *anti*-1-amino-3-[^{18}F]fluorocyclobutane-1-carboxylic acid (*anti*-3-[^{18}F]FACBC) which is a synthetic amino acid analog with little renal excretion and transport via sodium dependent and independent pathways [33-35]. In a recent study, *anti*-3-[^{18}F]FACBC demonstrated higher accuracy compared with ^{111}Indium-capromab-pendetide in the restaging of patients with suspected recurrent prostate carcinoma [36]. Yet there was suboptimal specificity in the prostate bed, especially after non-radical prostatectomy therapy. It is not yet clear if this was the result of sampling error during biopsy and/or nonspecific uptake by *anti*-3-[^{18}F]FACBC in non-neoplastic tissue.

In one study of patients with both primary and recurrent prostate carcinoma, DWMR demonstrated significant differences of mean apparent diffusion coefficients (ADC) between malignant and benign nodes with an accuracy of 85.6% (sensitivity 86%, specificity 85.3%) compared with an MR accuracy of 66.1% based on nodal size alone. Yet there was significant overlap of individual ADC values between benign and malignant foci [18]. In addition, less than 50% of patients had histologic follow-up and 6 month follow-up was accepted as truth in those without histologic confirmation. In another recent study in patients with suspicion of recurrence after high dose brachytherapy, DWMR was noted to have a sensitivity of 68% and a specificity of 95% in the detection of tumor in the prostate bed [37]. Multiparametric MRI achieved the highest sensitivity (77%) but with slightly decreased specificity (92%).

Thus, there may be utility in combining both multiparametric MR and synthetic amino acid PET as a hybrid transmolecular technique in the detection and restaging of recurrent prostate carcinoma. The data from this study could be used to design a more comprehensive study that would lend itself to novel PET-MR hybrid devices which are now commercially available.

There may also be genotypic and phenotypic differences between prostate carcinoma recurrence in the prostate bed and in extraprostatic nodal locations. Exome sequence analyses

of prostate cancer metastases and integrative copy number and gene expression analyses have identified mutations specific to specific metastases [7, 8]. Other authors have also identified important differences between primary and metastatic lesions including components of the AR pathways such as NCOA2 [9] and the polycomb complex chromatin suppressor EZH2 [10].

Furthermore, we have determined in RT-PCR analysis of primary and recurrent prostate carcinoma and distant metastases that the amino acid transport (AAT) proteins PAT1, LAT2 and ASCT2 as well as LAT1, xCT, LAT3 and ASCT1 are associated with malignancy. In analysis of lymph node metastases only, LAT1 and ASCT1 drop below significance. In addition, out of the AATs which were associated with malignancy, PAT1 had the highest correlation co-efficient of significance of expression to *anti*-3-[^{18}F]FACBC uptake [38].

A. Specific Aims

Aim 1. Investigate the ability of *anti*-[^{18}F]FACBC PET-CT and multiparametric/DW MRI imaging individually then together to detect local and extraprostatic recurrence of prostate cancer.

We will undertake a preliminary clinical trial with 25 patients post-definitive non-prostatectomy therapy for prostate carcinoma and who have suspected recurrence based on elevated PSA greater than nadir plus 2 ng/ml with absolute PSA ≥ 4.0 ng/ml with any doubling time (DT) or with PSA 2.0-3.99 ng/ml with DT ≤10 months. We will perform *anti*-[^{18}F]FACBC PET-CT with early and delayed imaging, as well as multiparametric/DWMR. Each study will be blindly interpreted then fused utilizing software registration and co-interpreted. Data from both studies will be used to direct biopsies to areas of concern in the prostate bed, and in extraprostatic locations. Results will be compared to the standard of pathologic analysis of prostate bed biopsies as well as clinical follow-up. The diagnostic performance of *anti*-[^{18}F]FACBC PET-CT and multiparametric/DWMRI will be assessed individually then together. We will also determine if there is correlation between synthetic amino acid PET and MR, such as SUV and apparent diffusion co-efficient (ADC).

Rationale for AIM 1.

As has been outlined in the Preliminary Results section of this proposal, *anti*-[^{18}F]FACBC has been demonstrated to 1) have high sensitivity in the detection of recurrent prostate carcinoma though with suboptimal specificity in the prostate bed, and 2) the ability to differentiate prostatic from extraprostatic recurrence with high accuracy. *anti*-[^{18}F]FACBC may thus have specific advantages for imaging recurrent prostate neoplasia within a clinical setting over conventional imaging. Yet, MR, especially with the addition of diffusion weighted imaging, has been reported in preliminary studies to provide high accuracy especially in the characterization of prostate bed recurrence. We hypothesize that utilization of a transmolecular approach combining synthetic amino acid radiotracer PET imaging with multiparametric MR has the potential to identify the presence and location of recurrent prostate carcinoma to a higher accuracy than either modality alone. **Therefore the most comprehensive manner to assess our hypothesis that PET-CT imaging with *anti*-[^{18}F]FACBC in combination with multiparametric MR will lead to improved patient care resulting from detection of local and distant recurrent disease (Specific Aim #1) is to undertake a prospective clinical trial designed to address these issues.**

Secondary Aims

Aim 2. To determine mRNA differences between prostate carcinoma recurrent in the prostate bed and extraprostatic locations and to correlate any difference with quantifiable imaging biomarker data such as SUV for *anti*-[^{18}F]FACBC PET-CT and ADC for MR.

Within the structure of the clinical trial (the same 25 patients) we will obtain sufficient tissue to perform mRNA sequencing on samples to exploit differences between the genetic profiles of recurrent prostate carcinoma in the prostate bed versus metastases. Since approximately 30% of patients with PSA > 4 ng/ml by our experience manifest with both prostate bed and nodal recurrence, we believe at least 8-10 patients in whom this comparison can be directly made will be available for analysis. This may also occur with lower PSA levels with faster DT. We will then correlate any differences in genotypic profile with imaging biomarkers from synthetic amino acid PET and MR, such as SUV and ADC.

Sub-aim 2a. To evaluate protein expression of previously identified amino acid transporters (AATs) that have been implicated in the uptake of anti-[18F]FACBC.

Also within the context of the clinical trial of 25 patients, we will, whenever there is sufficient tissue, determine if candidate amino acid transport (AAT) proteins identified from data obtained in an earlier study (Project 1: P-50 ICMIC) are expressed. We will perform IHC analysis of up to 7 candidate AAT or co-transporters, specifically PAT 1, LAT1, LAT2, 4F2hc, and ASCT2, LAT3 and ASCT1 to understand if the AAT whose mRNAs are increased in malignant prostate carcinoma and correlated with *anti*-[^{18}F]FACBC uptake are expressed at the protein level by IHC analysis.

Rationale for Aim 2 and Sub-aim 2a.

As has been outlined in the Preliminary Results section of this proposal, there is preliminary data in the literature of gene expression and mutational differences between primary prostate carcinoma cells and those prostate metastases [7-9] as well as between bone metastases and non-osseous metastases [8]. We propose within the context of this clinical trial to provide tissue to further understand these differences both in the ability of prostate carcinoma to transport and retain amino acids measured as uptake of *anti*-[^{18}F]FACBC and expression characteristic as studied by multiparametric MR such as ADC parameters on DWMR and uptake and retention of Gadolinium. If we can determine that there are gene expression differences between malignancy confined to the prostate bed and those that are extraprostatic, we can correlate these differences to PET uptake and MR appearance. Furthermore, we may also be able to derive incidental genotypic information that could be exploited on subsequent unrelated experiments. In addition, we have preliminary evidence based on our earlier research that PAT1, LAT2 and ASCT2 as well as LAT1, xCT, LAT3 and ASCT1 were associated with malignancy. Out of the AATs that were associated with malignancy, PAT1 expression had the strongest correlation coefficient relative to *anti*-3-[^{18}F]FACBC uptake [38]. We believe that a study such as this one provides an ideal opportunity to obtain both prostatic and extraprostatic tissue from the same patient in order to test these hypotheses using the patient as his own control.

Our rationale for utilizing patients with PSA ≥ 4 is as follows. A total of 127 patients have been studied already in an R-01 study of the diagnostic performance of *anti*-3-[^{18}F]FACBC versus conventional imaging. If the patients are stratified by PSA level as in the table below, 41/73 were positive in the prostate bed only, 25 in both prostate bed and lymph nodes, and 1 in lymph nodes only. Thus, in the 25 patients whom will be studied in this population,

approximately 23 will be expected to have disease in the prostate bed and approximately 9 will have disease in nodes, most of the latter will also have disease in the prostate bed. This should provide sufficient tissue for the above secondary aims. In addition, we have demonstrated that greater extraprostatic involvement will occur with shorter DT even at lower PSA levels. Thus the addition of PSA 2.0-3.99 ng/ml plus DT ≤ 10 months is a reasonable method to increase recruitment while still acquiring tissue from extraprostatic involvement.

PSA group	<4 (n=54)	≥ 4 (n=73)	Total(n=127)
Prostate bed only (+)	27	41	68
LN only (+)	3	1	4
Both	8	25	33
Total	38	67	105

We hypothesize that by identification of disease recurrence in the prostate bed and extra-prostatic locations, we can exploit differences in mRNA analysis to correlate these differences to synthetic amino acid transport as well as MR parameters such as ADC mapping, and also better understand the relationship of AAT protein expression to *anti*-3-[^{18}F]FACBC uptake. **Therefore the most comprehensive manner to obtain tissue to test our hypotheses is within the context of this prospective clinical trial.**

C1.0 Research Design and Methods (Aim 1)

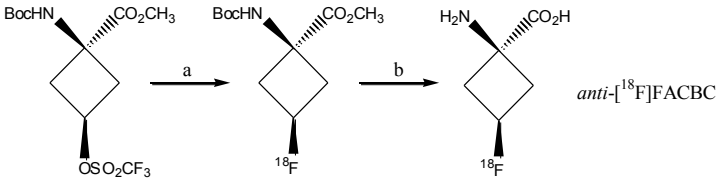
We will undertake a **clinical trial with 25 patients** who have undergone definitive therapy for prostate carcinoma and who have suspected recurrence based on elevated PSA greater than nadir plus 2 ng/ml with absolute PSA ≥ 4.0 ng/ml with any doubling time (DT) or with PSA 2.0-3.99 ng/ml with DT ≤10 months. We will perform *anti*-[^{18}F]FACBC PET-CT with early and delayed imaging as per the detailed protocol below. All patients will also undergo staging including multiparametric MR with diffusion weighted imaging. Other standard of care imaging such as bone scan, ProstaScint, CT, and transrectal ultrasound may also be performed per clinical judgment. These latter studies including MR are standard of care at our institution and will not be charged to the grant. We will investigate the ability of *anti*-[^{18}F]FACBC PET-CT imaging to detect recurrence of prostate carcinoma in the prostate bed and in extraprostatic locations validated by pathologic analysis of prostate bed biopsies ideally guided by MR or ultrasound fusion of molecular to anatomic data in the prostate bed and percutaneous or laparoscopic guided biopsies of nodal and skeletal disease. Within the structure of the clinical trial, we will also evaluate the ability of transmolecular imaging with *anti*-[^{18}F]FACBC PET-CT and multiparametric MR in the discrimination of prostatic from extra-prostatic recurrence, validated by a combination of pathologic analysis as well as imaging and clinical correlation. Though the grant itself extends for one year, patients may be followed up to 5 years to determine true negative imaging findings at minimal cost. Finally within the context of the clinical trial, tissue will be obtained for Aims 2 and Sub-aim 2a.

C1.1 Anti-[^{18}F] FACBC radiolabeling

Methods. Production will be accomplished by the GE FastLab cassette system. Alternatively by automated synthesis developed by J. McConathy and M.M. Goodman [39] as outlined in Figure 15. The automated radiosynthesis of *anti*-[^{18}F]FACBC will be carried out in a chemical process

control unit (CPCU) with a computer interface. The two-step reaction sequence will involve incorporation of no-carrier-added potassium [^{18}F]fluoride into a protected triflate precursor and deprotection using aqueous hydrochloric acid. The crude reaction mix will be passed in series through ion-retardation resin, an alumina-N SepPak®, an HLB cartridge and a 0.22 µm sterile filter, and the resulting aqueous solution will be collected in a dose vial. The radiochemical purity of the product will be determined by TLC. Additional chemical solvent purity will be measured by Gas Chromatography (GC). The total time for synthesis of *anti*-[^{18}F]FACBC after delivery of ^{18}[F]fluoride will be ~70 minutes, and the average decay-corrected yield of *anti*-[^{18}F]FACBC will be 24 ± 4 % (n = 40 runs, average ± standard error) in over 99% radiochemical purity. This procedure will provide 140-200 mCi of *anti*-[^{18}F] FACBC at the end of synthesis. We have prepared greater than 100 batch productions for tumor imaging in volunteer subjects.

Synthesis of *anti*-FACBC



a)K^{18}F, K_{222}, K_2CO_3; 90C b) 4 NHCl, 120 C

C1.2 PET-CT imaging protocol to measure amino acid uptake

Methods. PET-CT images will be acquired on a GE Discovery 690 Time of Flight (TOF) 16 slice PET-CT scanner or equivalent. All studies will use measured attenuation correction (routinely acquired through the initial CT portion of the scan). All subjects will be required to fast for four hours to normalize their neutral amino acid levels. Non-strict fasting is not an absolute contraindication and may be overridden on a case by case basis since it is unknown if fasting affects FACBC uptake positively or negatively. One hour prior to scanning, the patient will drink 500 ml of 1.2% (Readi-Cat) oral contrast over 1 hour to maximize conspicuity of abdomen and pelvic structures. IV contrast will not be used. Prior to placement in the tomographic gantry, an intravenous catheter will be placed for injection of tracer. The subject will be placed in the tomograph gantry for completion of the CT scan. Ambient conditions will be a quiet, dimly lit room. *anti*-[^{18}F]FACBC (10 mCi) will be injected into an antecubital vein in a slow bolus infusion over 1-2 minutes. At 5 minutes, 4 consecutive 2.5 minutes per frame acquisitions will be obtained starting from the prostate level of the pelvis and extending superiorly to the diaphragm. At 16 minutes, this process will be repeated. Thus, 4-15.5 minute, and 16-27.5 minute acquisitions from the pelvis extending superiorly to the abdomen at the diaphragm will be obtained (Figure 1). This imaging schema is based on analysis of our data to date in which early and delayed uptake to 30 minutes yields the most diagnostic information. Imaging may be carried out with HD mode and respiratory gating or TOF mode with or without respiratory gating.

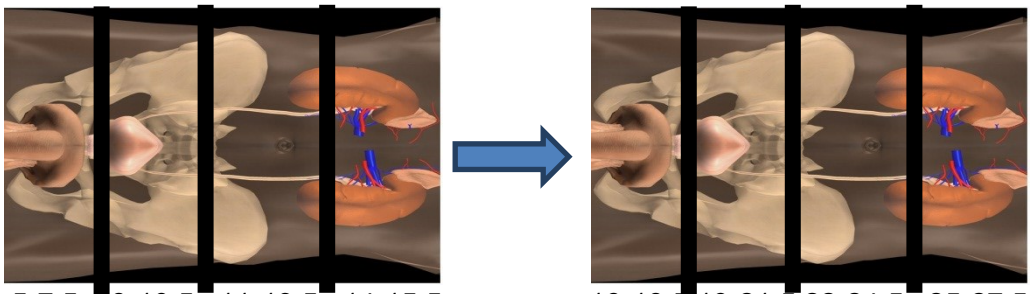


Figure 1: CT of abdomen and pelvis followed by injection of anti-[18F]FACBC then PET scanning as above.

Summary of PET-CT Scanning Procedure

1) The patient will be placed in the tomographic gantry for a CT scan of the abdomen-pelvis to be utilized for anatomic imaging and correction of emission data (approximately 1 minute).
2) The patient will then receive a bolus of *anti*-[^{18}F]FACBC injected IV over 1-2 minutes
3) The dosage will be approximately 10.0 mCi (3.70 x 108 Bq).
4) At 5 minutes after initial injection (3 minutes after injection ceases), a 2.5 minute per bed position PET acquisition will start at the pelvis with the inferior aspect of the acquisition to include the entire prostate or prostate bed.
5) 4 bed positions will be obtained which should cover pelvis through abdomen to the diaphragm.
6) This sequence will be repeated once.
7) The entire study including injection of radiotracer should take approximately 30 minutes.

C1.3 Multiparametric/DW MRI imaging

Pelvic and abdominal MRI through the prostate will be performed on a 1.5 T MR scanner or equivalent utilizing a standard clinical protocol (figure 2) including but not limited to the following sequences:
1) Dynamic contrast enhanced (DCE) perfusion protocol of the pelvis (including prostate) with delayed post-contrast sequences of the abdomen-pelvis.
2) High Resolution T2 of the pelvis
3) SPACE 3-D volumetric images of the pelvis
4) Diffusion-weighted imaging (DWI) of the abdomen and pelvis

Figure 2.

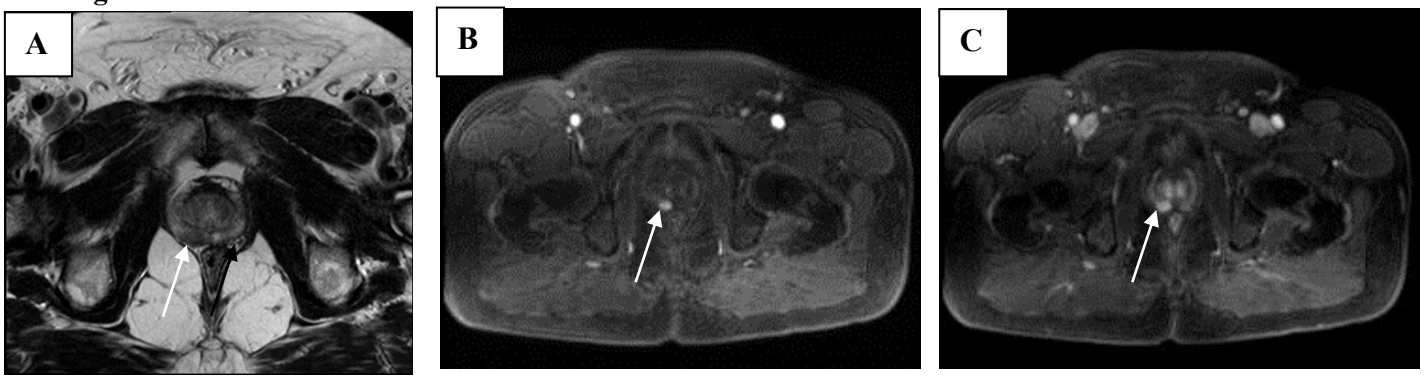


Figure 2. 8mm prostate carcinoma nodule in the right posterior peripheral zone (arrow) on high resolution T2 (A), dynamic 3D gadolinium enhanced T1 gradient echo 30 sec (B) and 90 sec MRI (C).

C 1.4 Image Analysis of *anti*-[^{18}F]FACBC PET-CT

Methods. The methods of image analysis to be used for the *anti*-[^{18}F]FACBC PET-CT are as follows:
1) Images will be reconstructed with iterative technique and hardware fused (PET to CT) on a MimVista or similar workstation which enables SUV (mean, maximum, total lesion activity) as well as standard size measurements of lesions. An edge seeking conformational volume of

interest tool as appropriate (PET Edge, MIMvista, Cleveland, OH) will typically utilized. If this is not possible due to anatomy an appropriate 2D or 3D ROI will be utilized.
2) Visual inspection of the PET-CT images by 2 board certified nuclear medicine imagers who will be blinded to all history and other imaging. Each reader will assess images individually and any disagreement will be resolved by consensus. Thus, Z score will be calculated.
3) For *anti*-3-[^{18}F]FACBC, uptake will be defined according to the following criteria in relation to background structures: mild (above blood pool but less than marrow), moderate (above or equal to marrow but less than liver), and intense (equal to or above liver). Visual analysis will aided by quantitative criteria of SUV_{max} lesion/SUV_{mean} background. Maximum and mean SUV of each focus of abnormal uptake as well as background structures including liver, marrow at L3, aorta, and bladder will be recorded. For prostate beds as well as extra-prostatic sites such as lymph nodes and bone, abnormal moderate or intense focal uptake over background marrow which persist from early to delayed images will be considered prospectively positive. These criteria were used to analyze data in our study of *anti*-[^{18}F]FACBC in recurrent disease [40].
4) Confidence in interpretation for disease within the prostate bed and outside the bed will be recorded with the following scale on a per patient basis and any all recordable lymph nodes: 1-defiinitvely negative; 2 -probably negative; 3 - indeterminate; 4 - probably positive; 5 – definitively positive.
5) In summary, we will record both visual and semi-quantitative analysis of the prostate bed, positive lymph nodes (greater than blood pool), and other structures such as skeletal foci on early and delayed time frames. We will use maximum and mean SUV as well as standard bidimensional size measurements. We will also record similar measurements on background structures such that we may derive uptake ratios.

C1.5 Image Analysis of *MR*

The MR studies will be interpreted by an MR imager. (At some point after primary analysis, a 2nd interpretation may also be blindly performed and agreement (Z Value) recorded.) Studies will be interpreted utilizing standard interpretative criteria for MR and including anatomic criteria, perfusion analysis and DWMR criteria. Confidence in interpretation for disease within the prostate bed and outside the bed will be recorded for each of these three interpretative categories individually and in aggregate with the following scale on a per patient basis and any all recordable lymph nodes: 1-defiinitvely negative; 2 -probably negative; 3 - indeterminate; 4 - probably positive; 5 – definitively positive. Typically, a combination of abnormality on all relevant sequences will result in a “5” (e.g. DCE, DWI, and T2), while no abnormality on any sequence will result in a “1”. A combination of suspicious and benign findings on pulse sequences will result in a “2”, “3”, or “4” depending on particular findings.

C1.6 Image Analysis of PET-MR

After individual interpretation of each study, then the PET and appropriate sequences from the MR (typically post-contrast abdominopelvic supplemented by other sequences) will be co-registered using rigid software registration on a MimVista 5.5 or similar workstation. The studies will then be analyzed by consensus by one of the 2 nuclear imagers and the primary research MR imager. All foci previously recorded by each group as positive or negative will then be assessed by the group taking into account all parameters previously employed. New foci may also be identified. Confidence in interpretation for disease within the prostate bed and outside the bed will then be recorded with the following scale on a per patient basis and any all recordable lymph nodes: 1-defiinitvely negative; 2 -probably negative; 3 - indeterminate; 4 - probably positive; 5 – definitively positive. Typically agreement on both studies will be recorded

as “1” or “5”, and combination of lack of agreement in a “2”, “3”, or “4” depending on particular findings.

C1.7 Clinical and Histologic Assessment.

Methods. After the *anti*-[^{18}F]FACBC scan has been obtained and interpreted, the following will occur:
1) All patients with identifiable sites of disease on either PET or MR imaging may undergo either ultrasound guided biopsy after fusion with PET and/or MR data (Fei R-01) or MR guided techniques as clinically appropriate. If this is not deemed appropriate by the clinical team and referring Urologist or Radiation Oncologist, patients will undergo standard TRUS 12-core biopsy as clinically appropriate. A 12 core biopsy protocol as clinically appropriate will be used as it has greater sensitivity for disease detection, with no increased morbidity.
2) Patients with abnormal suspicious findings on MR, and/or *anti*-[^{18}F]FACBC PET-CT may have those suspicious lymph nodes or other extraprostatic sites sampled as clinically appropriate through a combination of percutaneous image guided needle biopsy, laparoscopic techniques, as well as open lymph node dissection when appropriate. With both laparoscopic and open nodal dissections, it is standard practice to also sample adjacent imaging negative nodes. Rare sites of suspect metastases such as the inguinal nodal regions will be evaluated via a combination of physical exam and percutaneous biopsy as appropriate. On a per patient basis, suspicious uptake which corresponds to abnormal lymph nodes on MR or PET studies will be biopsied per standard of care as clinically appropriate.
3) All pathologic samples will undergo standard analysis to determine if prostate carcinoma cells are present as well as (if there is sufficient tissue) provide tissue for Aim 2 and Sub-aim 2a. Specimens will be fixed in neutral-buffered formalin, embedded in formalin, sectioned at 5-micron thickness and stained with hematoxylin & eosin (H&E) using standard pathology procedures. Board-certified anatomic pathologists from Emory University will perform all diagnosis of prostate carcinoma (and when applicable Gleason grading and staging) using standard criteria. RNA analysis and immunohistochemistry will be performed at end of study on parallel histologic sections of tissue confirmed to contain prostate carcinoma.
4) All patients may also be followed up as scientifically appropriate at least one year and up to five years regardless of initial findings in order to optimize correlation of imaging to clinical findings. It is not possible to do a template nodal dissection on all patients regardless of findings, and certainly not on nodal imaging negative patients. While it is true that we will not biopsy lymph nodes if all the imaging modalities are negative, there will be patients who are MR imaging positive but PET negative and visa-versa who will get biopsied. Yet biopsies or sampling will typically performed only when there is an identifiable target on anatomic imaging and will thus be standard of care. Some patients who undergo open or laparoscopic nodal sampling for positive findings will have a limited template dissection of adjacent imaging negative nodes (a standard practice). By clinical and ethical necessity, the imaging and biopsy negative patients will be confirmed by lack of lymph node progression on imaging as well as stable PSA levels. The standard of care outside the trial includes serial PSA measures as well as appropriate imaging such as CT or MR and repeat imaging as required.
5) A Ground Truth Panel composed of two urologists (Nieh and Master or substitute) and a radiation oncologist (Jani or substitute) will meet once sufficient data has been collected and/or communicated via email. The diagnostic imagers will not be included on this panel but will present the data. Truth will be ascertained via the criteria outlined below. If there were initial differences of opinion, further discussion will ensue until a group consensus can be achieved.

6) Presence or absence of disease in the prostate bed will be confirmed by biopsy only overridden by clinical data. For example, a substantial reduction in PSA after prostatic bed salvage therapy without a subsequent substantial PSA rise in at least 6 months would not only establish presence of presumed prostatic bed disease but also absence of extraprostatic disease. Extraprostatic nodal involvement per patient will be typically confirmed by pathologic proof or under certain circumstances by response after focal therapy if scientifically justifiable. Skeletal involvement will be confirmed with either biopsy or typical appearance on MR [41] or other accepted confirmatory imaging such as typical appearance on bone scan or CT. Absence of extraprostatic disease will typically be confirmed by either a substantial reduction in PSA after prostatic bed therapy without a substantial rise in at least 6 months as above, and/or stable appearance on CT or MRI for greater than 1 year without evidence of nodal or bone involvement as medically and scientifically appropriate. Because some patients had definitive follow-up for the prostate bed but not extraprostatic disease, and visa-versa, the number of patients in each sub-analysis may differ.
7) Patients who are prostate bed positive and extraprostatic negative may get treated with local therapy (radiation, cryoablation, salvage surgery, etc.) as appropriate. Most of these patients will not initially get hormonal therapy and will be entered into the followup group. It is true that some patients with either high Gleason scores, impending or with cord compression and/or pathologic fracture, hydronephrosis, and/or rapid PSA doubling time of less than 3 months will require hormonal therapy, but this is not the majority of patients (approximately 15-20% worst case scenario). The majority of patients (80-85%) will have low to intermediate risk disease and PSA recurrence reflecting the prevalence of low and intermediate risk disease in the United States. Therefore these patients can be followed off hormonal therapy but with local therapy as needed.

C1.8 Correlation of FACBC and MR to and Clinical Outcome

Upon completion of the study and/or after additional follow-up, each patient may be re-reviewed by the same Ground Truth Panel to determine if the predicted findings on *anti*-[^{18}F]FACBC scan and MR individually and in tandem match those of clinical and pathologic findings especially in regard to the identification of prostatic versus extraprostatic recurrence. Also at that time uptake parameters will be reviewed. At no time this study will interfere with proper therapy as determined by the patient's physicians and the patient.

C2.0 Study Design and Methods (Aim2 and Sub Aim 2a)

Rationale. We hypothesize that *anti*-[^{18}F]FACBC uptake is mediated by a LAT protein subtype or subtypes. However, the precise LAT subtype involved in all cases is unknown. We also further hypothesize that there are genotypic differences between recurrent prostate carcinoma cells confined to the prostate bed and those that are extraprostatic and that these differences can be explored via proper identification of candidate tissue via transmolecular imaging to be performed at termination of study or when sufficient tissue has been collected.

Experimental Design. To test the above hypothesis, we will compare mRNA expression of recurrent prostate carcinoma tissue within the prostate bed and extraprostatic. In addition we will utilize *anti*-[^{18}F]FACBC avid tissue to confirm that the following AAT subtypes based on preliminary data are expressed at the protein level: PAT 1, LAT1, LAT2, 4F2hc, and ASCT2, LAT3 and ASCT1. We will study 8-10 samples of each recurrent prostate carcinoma in the prostate bed and 8-10 samples of disease outside the prostate bed, preferably in the same patient. We predict that one or more AAT subtypes will be relatively overexpressed by IHC in FACBC-avid carcinoma. We also predict that there will be differences in mRNA expression

based on these tissue samples between disease confined to the prostate bed and those that are extraprostatic. Based upon tissue availability we will perform IHC in the following rank priority: First: PAT 1; Second: LAT1, LAT2, 4F2hc; Third: ASCT2, LAT3 and ASCT1. This priority ranking is based on our desire to first confirm phenotypic expression of PAT1, since presence of this AAT correlated most strongly with *anti*-[^{18}F]FACBC uptake, followed by LAT1 and LAT2 with the heterodimeric associated protein 4F2hc, and ASCT2, LAT3 and ASCT1.

C 2.1 Quantitative next-generation mRNA sequencing

Methods. For experiments on fixed tissue RNA will be extracted at the Cancer Genomics Shared Resource (CGSR) at the Winship Cancer Institute from macrodissected 5 micron sections. The CGSR has extensive experience in preparing Total RNA from FFPE tissues [42]. FFPE RNA extraction will be performed using the Omega Biotech methodology in 96-well format on a Kingfisher FLEX Liquid Handler Robot. FFPE RNA will be quantitated by nanodrop spectrophotometry, and tested for RNA integrity and quality by Taqman analysis of the RPL13a ribosomal protein. Samples with sufficient yield (>500 ng), A_{260}/A_{280} ratio > 1.8 and RPL13a C_T values less than 31 cycles will be used for preparing sequencing libraries using the TrueScript kit (Illumina). Amplified cDNA libraries will be sonicated to an average length of 250 bp with a Covaris E-120 Adaptive Focused Acoustics Instrument. We will repair the ends of the sonicated DNA using the Illumina TrueScript protocol and generate barcoded libraries for RNA-seq analysis on an Illumina HiSeq 2000 instrument at the Southern California Genotyping Consortium at UCLA. Agilent Bioanalyzer Nanochip analysis will be performed to ensure the integrity of libraries prior to sequencing. We will obtain 60 million reads per sample of 50bp paired-end sequencing reads using the Illumina version 3 kits with three samples multiplexed per sequencing lane.

Fluorogenic quantitative RT-PCR assays will be used to confirm changes identified by RNA-seq analysis and performed in triplicate with standard SYBR-Green methodology on the I-cycler system (Bio-Rad, Hercules, CA, USA) according to published protocols with minor modifications [43]. Reaction specificity will be assessed by melting point analyses, in which single melting point peaks are required at temperatures predicted by amplicon sequence. Reactions without reverse transcription and template will serve as controls for DNA contamination and specimen carry-over. Test gene expression will be normalized to 28S ribosomal RNA and referenced to a standard RNA specimen. Relative normalized gene expression of each LAT subtype will be compared in FACBC avid versus non-avid prostate carcinoma, with statistical significance assessed by analysis of variance (ANOVA).

C 2.2 Immunohistochemistry

Methods. Fixed tissue sections (5 µm thickness) will be dewaxed, and steam antigen retrieval will be performed at pH 6.5 in a pressure cooker for 20 minutes [44]. Tissue sections will be incubated with primary antibodies directed against PAT 1, LAT1, LAT2, 4F2hc, and ASCT2, LAT3 and ASCT1. Sections will be deparaffinized and subjected to heat-induced epitope retrieval by steaming for 15 minutes. IHC staining will be performed at the Winship Cancer Institute Research Pathology Shared Resource using an automated (DAKO) immunohistochemistry stainer and established procedures and protocols. Optimal dilutions for all antibodies will be determined empirically using archived sections from prostatectomies and blocking reagents recommended by the manufacturers. Immunohistochemical reactions will be developed with diaminobenzidine as the chromogenic peroxidase substrate. Sections will be counterstained with hematoxylin after immunohistochemistry. Specificity will be verified by negative control reactions without primary antibody. Intensity of the various immunohistochemical stains were scored blindly by a board-certified pathologist as follows; 0-

negative, 1+ (weak), 2+ (intermediate) and 3+ (strong). The expression of these markers will be correlated with FACBC uptake by ANOVA testing

C 2.3 RNA-Seq Results and Imaging Findings

RNA-Seq sequencing reads produced from the Illumina HiSeq 2000 will be mapped to the human reference genome (hg19) using Bowtie [45] and fragment reads per kilobase (FRPK) calculated using TopHat [46]. Only uniquely mapped reads will be retained for analysis. Relative changes in mRNA expression will be computed using Cufflinks and Cuffdiff [47] in a locally installed Galaxy bioinformatics environment [48, 49] deployed by the Moreno laboratory.

D. Subject recruitment:
All patients will be recruited from the Emory Healthcare (Drs. Nieh, Master, Rossi and Jani), Urology and Radiation Oncology clinics per the inclusion and exclusion criteria below.

Inclusion Criteria:

1. Patients must be 18 years of age or older.
2. Patients will have been originally diagnosed with localized (Stage T1c, T2, or T3) prostate carcinoma and have undergone what was considered definitive non-prostatectomy therapy for localized disease.
3. In the case of cryotherapy, external beam radiation, or HiFU the procedure will have occurred at least one year in the past. In the case of brachytherapy, treatment will have occurred at least 2 years in the past to eliminate patients with so-called “PSA bump.”
4. Patient will have suspicion of recurrent prostate carcinoma as defined by: the ASTRO-RTOG Phoenix criteria of elevated PSA greater than nadir plus 2 ng/ml with absolute PSA ≥ 4.0 ng/ml with any doubling time (DT) or with PSA 2.0-3.99 ng/ml with DT ≤10 months.
5. Ability to lie still for PET scanning
6. Patients must be able to provide written informed consent.

Exclusion Criteria:
1. Age less than 18.
2. Greater than T3 disease in past and/or treated with prostatectomy.
3. Less than 1 year since cryotherapy, external beam radiation therapy, or HiFU, or 2 years since brachytherapy.
4. Does not meet above criteria of suspicious PSA elevation
5. Inability to lie still for PET scanning
6. Cannot provide written informed consent.
7. Bone scan findings characteristic for metastatic prostate carcinoma
8. Less than 1 month since any prior prostate biopsy (to decrease false positive uptake from inflammation).

Written informed consent will be obtained before subject participation in the study. No procedures will be performed before written informed consent is obtained. Participants will be assigned an identification number for screening purposes; data collected during the screening process will be recorded using that number.

PET Scan Day: The following procedures will be performed:

- **Obtain written informed consent**
- **Inclusion/exclusion criteria review**
- **Vital signs measurements**
- **Clinical laboratory tests (serum chemistries, CBC and urinalysis)**
  - **To be repeated at one week (+/- 3 days) after scan**

E. Statistical Analysis

E.1 Aim 1

Receiver Operating Characteristic (ROC) analysis will be conducted to instigate the predictive power of FACBC PET and MR and determine if a variation in standard criteria or if combined criteria better maximizes sensitivity, specificity and accuracy of *each technique* using Chi-square test. The predictive accuracy of each technique will be determined using the positive predictive value (PPV) relative to the clinical and pathologic findings. The PPV of imaging will be assessed by calculating the probability of obtaining clinical and pathological outcomes that confirm the predicted positive findings based on the individual and combined scans. A similar negative predictive value (NPV) will also be calculated based upon a negative pathologic sampling as well as clinical/imaging follow-up at least 1 year or more. Formal hypothesis testing will be conducted to obtain inferences regarding differences between the predictive accuracies of these two approaches.

Each case will be evaluated as follows:

1) Results of biopsy as well as clinical follow-up will be correlated with prediction of local and distant neoplastic involvement by MR and *anti*-[^{18}F]FACBC PET-CT.
2) Results of biopsy of lymph nodes or other extra-prostatic sites as well as clinical follow-up will be correlated with prediction of extraprostatic neoplasia by MR and *anti*-[^{18}F]FACBC PET-CT.
3) Registered PET and MR will be correlated both qualitatively and quantitatively to pathologic and clinical results and in the discrimination of prostatic from extra-prostatic recurrence.
4) **Thus, truth for presence of disease will be established by biopsy/pathology and/or appropriate clinical followup. Truth for absence of disease will be established via pathologic sampling as well as standard clinical follow-up for at least 6 months and up to five years. We believe there will be enough patients with biopsy and clinically followed-up negative lymph nodes to calculate a true negativity rate.**

E2.0 Secondary Aims

Aim 2. and **Sub-aim 2a.**
Depending on the normality of data distribution, t-test or Wilcoxon rank sum test will be conducted to compare the mRNA differences, genomic differences in gene expression, alternative splicing, indels and single base mutations between prostate carcinoma recurrent in the prostate bed and extraprostatic samples. Analysis of variance (ANOVA) will be used to compare the IHC staining of protein levels between the different categories of FACBC uptake. Tukey pairwise comparison will be further conducted when the overall comparison with ANOVA

is significant at the significance level of 0.05. Pearson/Spearman correlation coefficients will be estimated to measure the associations: 1) between mRNA levels of candidate AAT genes and FACBC uptake; 2) between mRNA differences and quantifiable imaging biomarker data such as SUV for *anti*-[^{18}F]FACBC PET-CT and ADC for MR. Multivariate analyses will further be conducted, in which general linear model (GLM) will be employed to assess the adjusted relationships mentioned above after adjusting for the other covariates.

Sample size and power calculation

The sample size of this study is 25 patients. The power calculation is based on the comparison of mRNA differences between the prostate bed and extraprostatic samples using two sided t-test. At the significance level of 0.05, the sample size of 25 will achieve a power of at least 80% to detect one standard deviation or higher difference in mRNA difference between the two groups of samples.

F. Adverse Event Reporting

An adverse event is defined as any untoward medical occurrence associated with the use of a drug in humans whether or not considered drug-related

A significant shift from baseline which can be attributable to the radiotracer injection and not the patient's medical condition will be considered an unexpected AE. An event greater than 7 days post scan will not be considered an AE since 95% of ligand is eliminated by 7days.

The National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE) Version 4.03 (June 14, 2010) will be used as a guide address potential AEs subject to limitations above and medical and scientific judgment as to plausibility of such criteria in a diagnostic radiotracer study.

<u>Adverse Event Reporting</u>
Any patient death that may be due to the study procedure (i.e. severe radiotracer reaction), unanticipated problem, would be promptly reported to the Emory IRB office. Additionally any patient death not associated with the study procedure or serious unanticipated event(s) (i.e. radiotracer allergy) will be reported to the Emory IRB and FDA upon continuing review. Protocol deviation/non-compliance will be reported according to IRB Policies & Procedures. This radiotracer is studied under IND 72437 and monitoring will be performed per already agreed upon FDA guidance. Over 900 patients in multiple centers have been studied without adverse events.

G. Safety Monitoring

This study is being performed under the auspices of FDA IND 72,437. Patients will be monitored by the technologists and study nurse before and after the studies for any adverse events/reactions. They will be given contact phone numbers to call if they experience any problems (i.e. problems with the IV site, any allergic reaction symptoms). They will be followed routinely by their referring physician with clinical exams, and the PI will work with the co-investigators and referring physicians to ensure that the patients continue to follow up as

scheduled. In addition, a DSMB who will consist of Drs. Nieh, Schuster, Halkar, and Master will meet formally if a drug related serious adverse event has been reported.

Patient safety, study efficacy and compliance will be reviewed at the Urology working group meeting. The Data and Safety Monitoring Committee (DSMC) of the Winship Cancer Institute will also oversee the conduct of this study (every 6 months or annually – depending on the risk level of the protocol). This committee will review pertinent aspects of study conduct including patient safety, compliance with protocol, data collection and efficacy. The committee will review the charts of 10% of patients enrolled to the study and two of the first 5 patients entered to the study. The Committee reserves the right to conduct additional audits if necessary. The Principal Investigator (PI) or designee is responsible for notifying the DSMC about the accrual of patients when the first 5 have been entered to the study. The PI or designee will also notify the DSMC of study status within 2 months before the next scheduled review is due.

Any serious adverse events will be communicated by the PI to the Emory IRB and FDA using standard adverse event reporting forms. Yearly safety reporting will also be forwarded to the FDA. A formal DSMB will not be utilized as this is a diagnostic study with minimal risk to the patient.

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