^{18}F-FSPG PET/CT and Integrated Biomarkers for Early Lung Cancer Detection in Patients with Indeterminate Pulmonary Nodules

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IRB-46607 **LUN0106** **NCT03824535** **IND 125516**

Version date: 08 June 2023

Initial SRC Approval Date – 30 October 2018

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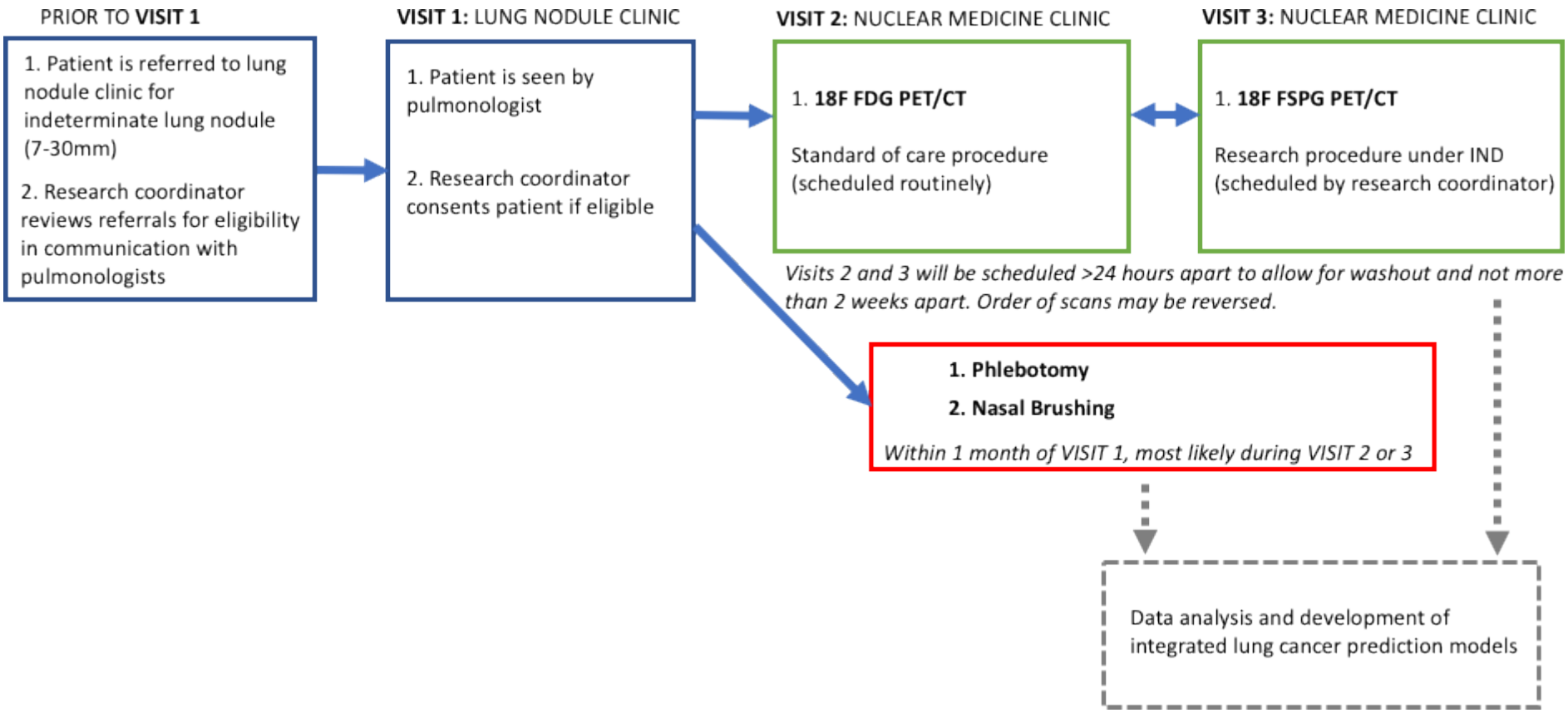
SCHEMA



- All PET imaging studies and image analysis will take place at Stanford (total of 120 patients).
- Nasal brushings and blood samples will also take place at Stanford according to our own protocols. Eventually these samples will be combined with other cohorts (total of > 900 patients) for collective analysis with our collaborators at Boston University.

LIST OF ABBREVIATIONS AND DEFINITION OF TERMS

CBC	Complete blood count
CI	Confidence interval
CRF	Case report/Record form
CTCAE	Common Terminology Criteria for Adverse Events
DSMB	Data Safety Monitoring Board
18F	Fluorine-18
FDG	Fluorodeoxyglucose
FSPG	(4S)-4-(3-18F-Fluoropropyl)-L-Glutamate
IND	Investigational New Drug
IRB	Institutional Review Board
IV	Intravenous
LDCT	Low dose computed tomography
NPV	Negative Predictive Value
PET/CT	Positron emission tomography – computed tomography
PPV	Positive Predictive Value
ROC	Receiver-Operative-Characteristic
SAE	Serious adverse event
SUV	Standard Uptake Value

1. OBJECTIVES

1.1. Primary Objective

Comparison of ^{18}F-FSPG accumulation with ^{18}F-FDG accumulation to assess whether (4S)-4-(3-18F-Fluoropropyl)-L-Glutamate (^{18}F-FSPG) PET is better at discriminating between benign and malignant nodules.

1.2. Secondary Objective

To develop and validate early lung cancer detection biomarkers that would directly impact the growing need to integrate imaging and non-invasive molecular diagnostics for indeterminate pulmonary nodules and allow physicians to avoid unnecessary invasive procedures in patients with benign lung disease. We will work with collaborators at Boston University to develop prediction models that could identify patients with lower likelihood of malignancy who can be followed by CT surveillance, instead of invasive lung biopsy.

2. BACKGROUND

2.1. Study Disease

Lung cancer remains the leading cause of cancer death, in large part due to our inability to intercept the disease process prior to its progression to an advanced state. Cancer interception refers to actively interrupting the cancer development process and decreasing the rate of clinical presentation with advanced disease. The rate-limiting steps impeding effective lung cancer interception are 1) a lack of understanding of the earliest targetable molecular events in lung carcinogenesis; 2) the challenge of developing highly sensitive and specific methods for early detection; and 3) the lack of markers for predicting or monitoring the efficacy of interception approaches. The USPSTF recommends annual screening for lung cancer with low-dose computed tomography (LDCT) in adults aged 55 to 80 years who have a 30 pack-year smoking history and currently smoke or have quit within the past 15 years. With the LDCT screening a growing number of lung nodules is being detected. The nodule size range in screen-eligible individuals generally represents intermediate risk where there is considerable clinical uncertainty about diagnostic approach. A sensitive biomarker with high negative predictive value would enable physicians to avoid unnecessary procedures in patients with benign lung disease, avoiding substantial risks and costs.

2.2. Imaging agent

While other radiopharmaceuticals are available, ^{18}F-fluorodeoxyglucose (^{18}F-FDG) is still the primary radiotracer clinically used for the evaluation of malignancies with PET/CT. ^{18}F-FDG allows the non-invasive visualization of the glycolytic pathway in cells. While effective for many cancer types, it also has many limitations including poor uptake in certain cancer types, prominent background uptake in the brain, kidneys, and often the gastrointestinal tract, and prominent uptake in benign inflammatory or infectious processes.

New insights into other metabolic pathways utilized by tumor versus normal cells have led to the development of a new classes of cancer diagnostics and therapeutics. Many tumor types are dependent on the glutaminolytic pathway. After glutamine enters the cell,

glutaminase converts it to glutamate, which is subsequently used in several metabolic pathways in the cancer cell, including as a substrate for the Krebs Cycle and for redox reactions. This pathway is an alternative to the better-known glycolytic pathway mentioned above. Some cancers that depend on glutamine as an energy source demonstrate high levels of glutaminase expression and a high intratumoral ratio of glutamate-to-glutamine. Other glutamine-dependent tumors have normal glutaminase activity but other changes that may result in increased glutamine utilization. Lung cancers are described to be dependent on the glutaminolytic pathway (1).

(4S)-4-(3-[^{18}F]fluoropropyl)-L-glutamate (^{18}F-FSPG, prior alias BAY 94-9392) is a novel agent for PET imaging and is specifically transported into cells via system xC-. This exchanger binds the amino acids glutamate and cystine with similar affinity and is able to transport both into the cell by exchange of abundant intracellular glutamate. Intracellular cystine is rapidly reduced to two molecules of cysteine, which are either used in glutathione (GSH) biosynthesis or contribute directly to redox maintenance. The ^{18}F-FSPG molecule, as a glutamate analog, is perfectly suited to provide important non-invasive information about tumor biology that is uniquely different from anatomic imaging such as CT or MRI and from ^{18}F-FDG. ^{18}F-FSPG has been shown to be taken up by non-small cell lung cancers (2). Our aim is to evaluate this imaging agent in the indeterminate lung nodule setting and determine if it can be used to identify early lung cancers.

2.3. Clinicaltrials.gov

This study is registered at ClinicalTrials.gov.

2.4. Rationale

There is a growing need for markers to identify the small number of malignant pulmonary nodules from the large number of benign nodules identified by chest CT. We propose to develop integrated clinical, molecular and imaging-based models for lung cancer in older smokers with nodules 7 to 30 mm at elevated risk for lung cancer based on lung cancer screening eligibility criteria. These indeterminate pulmonary nodules (IPNs) may have been detected in the context of screening or incidentally as part of routine practice. While a number of clinical risk models exist (3, 4), this nodule size range in screen-eligible individuals generally represents intermediate risk where there is considerable clinical uncertainty about diagnostic approach. A sensitive biomarker with high negative predictive value would enable physicians to avoid unnecessary procedures in patients with benign lung disease, avoiding substantial risks and costs. Our hypothesis is that we can improve current lung cancer detection by incorporating detailed information about nodule imaging characteristics and molecular data from surrogate tissues (nose and plasma) into risk models. The key advances over our prior and ongoing funded work are 1) detection of tumor-associated mutations in plasma; 2) focusing on complete sequencing-based characterization of the nasal transcriptome; 3) performing single-cell RNA-seq of nasal epithelium to identify the cellular architecture of cancer-associated expression; 4) incorporating imaging characteristics of the nodule uncovered by advanced semantic and computational methods; 5) piloting study of ^{18}F-FSPG PET tracer; and 6) incorporating each of these data types together with clinical data into an algorithm that can trigger appropriate diagnostic workup of IPN patients.

2.5. Preliminary results

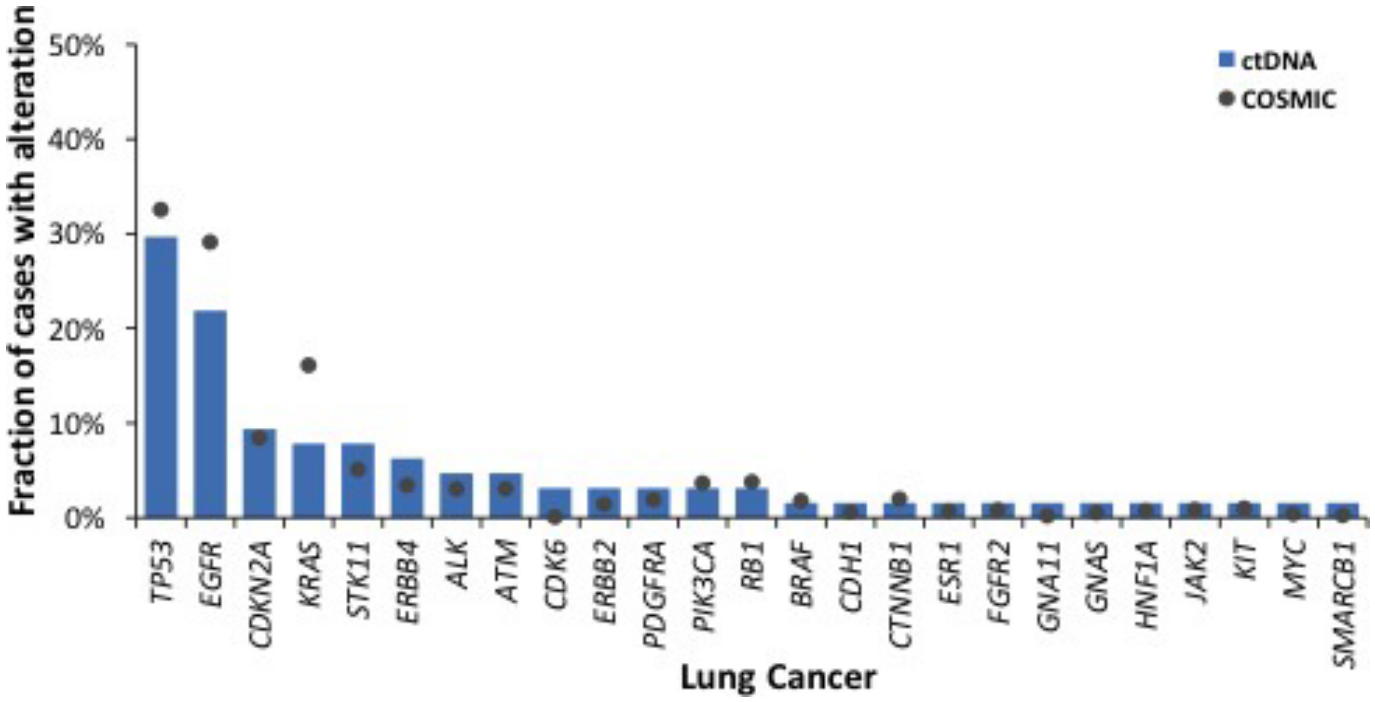


Figure 1. Mutation frequencies in cfDNA of lung cancer patients. The ctDNA mutational frequencies (bars) are similar to previously reported tumor mutation frequencies (dots) from COSMIC for 76 out of 81 genes analyzed (P > 0.05 for 76 of 81 genes,

Detection of mutations in circulating tumor DNA. We have used both targeted and whole genome methods (5) to identify genomic alterations in the plasma of healthy individuals and patients with cancer. Most recently, we have used the TEC-seq approach described above to examine plasma specimens from 44 healthy individuals: no mutations were observed in the cancer driver genes analyzed in our panel. We next analyzed plasma samples from lung cancer (n = 64), breast cancer (n = 46), colorectal cancer (n = 42), and ovarian cancer patients (n = 42). These untreated patients were largely diagnosed at stage I and II. We found that the concentration of ctDNA in plasma from cancer patients was ~29 ng/mL, significantly higher than that observed in healthy individuals (n = 44; 7 ng/mL; P = 0.001). By TEC-seq, we detected cancer driver mutations in plasma from over half of stage I and II colorectal, ovarian, lung, and breast cancer patients. The affected genes and mutation frequencies in the lung cancer patient plasma was similar to what has previously been reported (**Figure 1)**. On average, 2.1 alterations were observed in each patient with detectable ctDNA, with lung cancers having a higher number of alterations per case. We next evaluated 216 alterations in 100 patients where matched tumor tissue and blood cells were available. Overall, 82 of the 100 patients (82%) had at least one alteration observed in the circulation that was identical to that in the tumor specimen. We therefore expect that the fraction of cancer patients with a ctDNA-detected mutation will increase as the TEC-seq panel is expanded to include more cancer-associated mutations.

Nasal epithelial gene expression differences associated with lung cancer. We have previously established the feasibility of detecting lung cancer via nasal epithelial gene expression (6) using nasal microarray data from current or former smokers undergoing bronchoscopy for suspect lung cancer (as part of our validation of the Percepta™ bronchial genomic test for lung cancer) (7, 8). We derived a 30-gene nasal biomarker that in an independent test set significantly improved the performance of a clinical risk model for lung cancer (6). This proof-of-concept nasal biomarker was developed in the clinical setting of bronchoscopy, potentially enriching for higher risk patients with larger lesions. The important next step is to build a biomarker in the clinical setting of all indeterminate lung nodules and to incorporate additional molecular and imaging features.

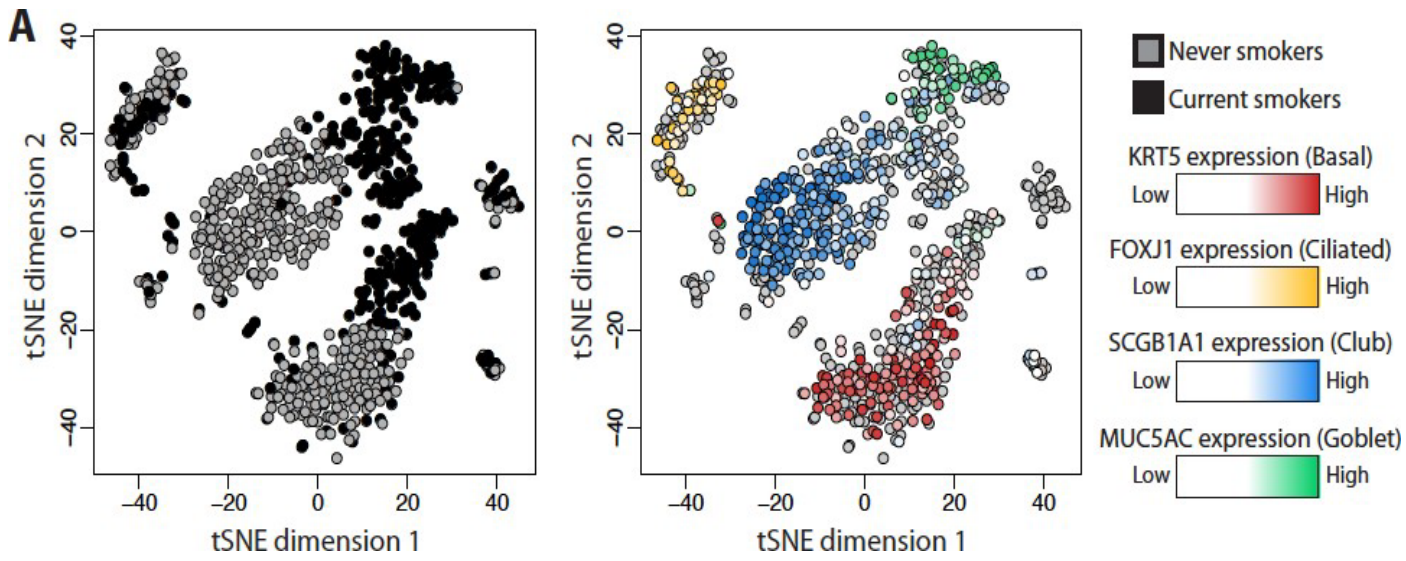


Figure 2. Single-cell RNA-seq of the normal bronchial epithelium. t-SNE clustering of single cell transcriptomes from 6 current and 6 never smokers. Left: Cells are colored by smoking status, with black cells from smokers and grey cells from nonsmokers. Right: Cells are colored by the expression levels of known cell type markers.

Single cell RNA-seq of bronchial airway epithelium. To explore the value of single cell transcriptomics in understanding smoking-related airway injury, we performed scRNA-seq of ~1200 cells from 6 healthy current and 6 never smokers using CEL-Seq (9). Global clustering using t-SNE indicates cell subsets enriched for known airway cell-type markers (**Figure 2**). Goblet cells (MUC5AC$^+$) are significantly more abundant in current smokers, whereas basal (KRT5$^+$) and club cells (SCGB1A1$^+$) are significantly more abundant in the airways of never smokers. To explore lung-cancer associated airway gene expression, we have performed scRNA-seq on ~1200 cells from 12 patients undergoing bronchoscopy for suspect lung cancer. We find expression of many genes in our bronchial airway gene expression biomarker (7) localizes to t-SNE cell clusters, suggesting that biomarker performance might be enhanced by assessing specific airway cell types.

Semantic and quantitative imaging variables for diagnostic risk of lung cancer. Dr. Aberle has developed logistic regression models combining clinical and semantic imaging variables in 11,128 CT-detected nodules from the NLST and found that semantic imaging features significantly improves the performance of clinical risk models of disease in cross validation

from 0.76 (95% CI: 0.74-0.79) to 0.89 (95% CI: 0.87-0.90). In separate work, Dr. Aberle has identified quantitative imaging features in 146 ever smokers aged 50 to 80 years with incidentally-detected nodules from the UCLA EMR with high prediction performance (AUC-.86) in cross validation. Our preliminary data establishes the potential of both semantic and quantitative feature-based models for lung cancer.

	FSPG	FDG
Control	1.83±0.92	6.95±3.47
Case	7.52±6.68	14.35±6.03

Table 1. SUV_{max} with standard deviation for non-malignant lung

^{18}F-FSPG PET tracer. Hijacking energy metabolism is a hallmark of cancer (10). ^{18}F-FSPG is a glutamate analogue tracer that is transported into cells via the system x_C– transporter, which maintains the cellular redox environment (11). This transporter is also important in tumor growth, progression and treatment resistance (12, 13). ^{18}F-FSPG visualizes distinct tumor biology compared to ^{18}F-FDG, which measures cellular glucose uptake (14). Studies at Stanford have shown that lung cancer is well visualized by ^{18}F-FSPG with low background uptake (2, 15). New data suggest that ^{18}F-FSPG has lower uptake in many inflammatory conditions (16), which can severely limit ^{18}F-FDG PET accuracy (**Table 1)**. We therefore hypothesize here that ^{18}F-FSPG is a superior tracer to assess the likelihood of cancer in pulmonary nodules.

Relevance of preliminary data: The preliminary data above establishes 1) our ability to measure a panel of mutations in ctDNA in early stage lung cancer, setting the stage for evaluating the panel as an early detection tool in the IPN setting; 2) nasal gene-expression as a potential diagnostic for lung cancer; 3) our ability to perform scRNA-seq of airway epithelial brushings in order to characterize the heterogeneity of the field; 4) semantic and quantitative imaging markers as diagnostic in the IPN setting; 5) the potential for novel molecular imaging markers to improve upon existing PET markers.

2.6. Study Design

120 patients with indeterminate lung nodules will be enrolled in the study at Stanford. Blood and nasal brushings will be collected once. The samples from these 120 patients will be part of a bigger cohort of over 900 patients for collaborative data analysis.
All samples will be de-identified before shipment or other data sharing with collaborators takes place. An additional 70 patients who may or may not have smoking history will be imaged at Stanford and analyzed with the other 50 patients enrolled at Stanford.

All 120 patients will be imaged once by ^{18}F-FSPG PET/CT as part of this open-label, nonrandomized, single-dose explorative study and once by ^{18}F-FDG PET/CT. PET/CT imaging will be scheduled > 24 hours apart to allow for imaging agent washout, and not more than two weeks apart to allow for optimal image comparison.

Clinical diagnosis (cancer / benign disease) is based on 1) histopathology of biopsy/surgical specimen or 2) surveillance by CT scans for a period of 2 years to confirm that there is no

growth of the nodule. All patients without histopathological diagnosis will undergo CT surveillance for a 2-year period by clinical standards.

3. PARTICIPANT SELECTION AND ENROLLMENT PROCEDURES

Inclusion and Exclusion Criteria are provided on the Eligibility Checklist, following, and which may be extracted for use in screening potential subjects.

The following Participant Eligibility Checklist will be completed in its entirety for each subject prior to registration. The completed, signed, and dated checklist will be retained in the subject's study file, and the study's Regulatory Binder.

Pursuant to Stanford Medicine SOP "Confirmation of Participant Eligibility in Clinical Trials," the treating Physician (investigator); the Study Coordinator; and an Independent Reviewer will verify that the subject's eligibility is accurate; complete; and legible in source records. A description of the eligibility verification process will be included in the EPIC or other Electronic Medical Record progress note.

3.1. Participant Eligibility Checklists

For each prospective study participant that is screened, the appropriate checklist will be printed, the results recorded, and filed in the respective subject binder or file. It is anticipated that not all prospective study participants will be enrolled.

Eligibility Checklist (Group 1: Subjects with smoking history)

I. Protocol Information

Protocol Title:	18F-FSPG PET/CT and Integrated Biomarkers for Early Lung Cancer Detection in Patients with Indeterminate Pulmonary Nodules
Group	Group 1: Subjects with smoking history
eProtocol number: **OnCore number:**	IRB-46607 LUN0106
Principal Investigator:	Carina Mari Aparici, MD

II. Subject Information

Subject name / Unique ID:	/	
Gender	☐ Male	☐ Female

III. Study Information

Inclusion Criteria

Prospective Participant Must MATCH ALL these Inclusion Criteria to be Eligible	**Yes**	**No**	**Supporting Documentation ***
1. Pulmonary nodule between the size of 7 to 30 mm. Subjects with multiple nodules may be eligible if the dominant nodule is 7 to 30 mm.	☐	☐	
2. ≥ 45 years old	☐	☐	
3. Current or former cigarette smoker, with ≥ 20 pack years	☐	☐	
4. Documented informed consent	☐	☐	

Exclusion Criteria

Prospective Participants Must NOT Match ANY of These Exclusion Criteria	Yes	No	Supporting Documentation *
1. History or previous diagnosis of lung cancer	☐	☐	
2. Any other current or previous malignancy within the past 5 years other than exceptions as determined by the opinion of the treating MD and PI, which include: • Adequately treated basal cell or squamous cell skin cancer • Carcinoma in situ of cervix • Prostate cancer with stable PSA level for >3 years, or • Other neoplasm that, in the opinion of the treating MD and PI, will not interfere with study-specific endpoints	☐	☐	
3. Pregnant or nursing (per department of radiology policy)	☐	☐	

* All subject files must include supporting documentation to confirm subject eligibility. The method of confirmation can include, but is not limited to, laboratory test results, radiology test results, subject self-report, and medical record review.

V. Statement of Eligibility

By signing this form of this trial I verify that this subject is: ☐ eligible / ☐ ineligible for participation in the study. This study is approved by the Stanford Cancer Institute Scientific Review Committee, the IRB of record, and has finalized financial and contractual agreements as required by Stanford School of Medicine's Research Management Group.

Study Coordinator printed name:	Date:
Signature:	

Investigator printed name:	Date:
Signature:	

Triple-check reviewer printed name:	Date:
Signature:	

Eligibility Checklist (Group 2: Smokers and Non-Smokers)

I. Protocol Information

Protocol Title:	18F-FSPG PET/CT and Integrated Biomarkers for Early Lung Cancer Detection in Patients with Indeterminate Pulmonary Nodules
Group	Group 2: Smokers and non-smokers
eProtocol number: **OnCore number:**	IRB-46607 LUN0106
Principal Investigator:	Carina Mari Aparici, MD

II. Subject Information

Subject name / Unique ID:		/
Gender	☐ Male	☐ Female

III. Study Information

Inclusion Criteria

Prospective Participant Must MATCH ALL these Inclusion Criteria to be Eligible	**Yes**	**No**	**Supporting Documentation ***
1. Pulmonary nodule between the size of 7 to 30 mm. Subjects with multiple nodules may be eligible if the dominant nodule is 7 to 30 mm.	☐	☐	
2. ≥ 45 years old	☐	☐	
3. Documented informed consent	☐	☐	

Exclusion Criteria

Prospective Participants Must NOT Match ANY of These Exclusion Criteria	Yes	No	Supporting Documentation *
1. History or previous diagnosis of lung cancer	☐	☐	
2. Any other current or previous malignancy within the past 5 years other than exceptions as determined by the opinion of the treating MD and PI, which include: • Adequately treated basal cell or squamous cell skin cancer • Carcinoma in situ of cervix • Prostate cancer with stable PSA level for >3 years, or • Other neoplasm that, in the opinion of the treating MD and PI, will not interfere with study-specific endpoints	☐	☐	
3. Pregnant or nursing (per department of radiology policy)	☐	☐	

* All subject files must include supporting documentation to confirm subject eligibility. The method of confirmation can include, but is not limited to, laboratory test results, radiology test results, subject self-report, and medical record review.

V. Statement of Eligibility

By signing this form of this trial I verify that this subject is: ☐ eligible / ☐ ineligible for participation in the study. This study is approved by the Stanford Cancer Institute Scientific Review Committee, the IRB of record, and has finalized financial and contractual agreements as required by Stanford School of Medicine's Research Management Group.

Study Coordinator printed name:	Date:
Signature:	

Investigator printed name:	Date:
Signature:	

Triple-check reviewer printed name:	Date:
Signature:	

3.2. Informed Consent Process

All participants must be provided a consent form describing the study with sufficient information for participants to make an informed decision regarding their participation. Participants must sign the IRB approved informed consent prior to participation in any study specific procedure. The participant must receive a copy of the signed and dated consent document. The original signed copy of the consent document must be retained in the medical record or research file.

Study Timeline

3.2.1. Primary Completion:

The study will reach primary completion 36 months from the time the study opens to accrual.

3.2.2. Study Completion:

The study will reach study completion 60 months from the time the study opens to accrual.

4. IMAGING AGENT AND PROCEDURE INFORMATION

4.1. Imaging Agent and Imaging Procedure

Patients enrolled in this study will undergo a one-time ^{18}F-FSPG PET/CT procedure which includes a single IV injection of ^{18}F-FSPG. Patients will also undergo one ^{18}F-FDG PET/CT as part of their standard of care. The two PET/CT procedure will be scheduled > 24 hours apart to allow for imaging agent washout, and not more than two weeks apart.

The nuclear physician or designee is responsible for ensuring that deliveries of the imaging agent and other materials from the manufacturing site are correctly received, recorded, handled, and stored safely and properly in accordance with all applicable regulatory guidelines, and used in accordance with this protocol.

Unless otherwise agreed, imaging agent vials must be destroyed on-site after use. The transport containers have to be returned to the manufacturing site. A list of imaging agent or other materials that were returned, or destroyed, must be prepared and signed by the principal investigator or designee. If there are any discrepancies, an explanation for these should also be provided

Physical, Chemical and Pharmaceutical Properties of the Imaging Agent

IUPAC name: (S)- 4-(3-[^{18}F]Fluoropropyl)-L-glutamic acid

Chemical name: (2S, 4S)-2-Amino-4-(3-[^{18}F]-fluoro propyl) pentane dioic acid

Empirical formula: $C_8H_{14}FNO_4$

Molecular weight: 206.2 g/mol

The active pharmaceutical ingredient for PET/CT imaging is the compound ^{18}F-FSPG that is labeled with the radioactive fluoride isotope ^{18}F. ^{18}F has a half-life of 110 minutes. The radioactive substance and the final imaging product are produced on-site at the study site

according to the radiolabeling and purification procedure described in the site Standard Operating Procedures (SOPs) and the Investigational New Drug application (IND). The Department of Chemistry, Manufacturing, Controls (CMC) personnel of Stanford will train qualified radiochemists/pharmacists with respect to the imaging agent production and handling. The precursor PI-020 and a non-radioactive standard used as a reference for Quality Assurance, and corresponding Certificates of Analysis will be provided by Piramal Imaging, SA.

Each batch of ^{18}F-FSPG produced must meet criteria listed in the specification for identity, purity, concentration, specific activity and pH before being released. Manufacturing and quality control testing will be checked and documented accordingly. Sterility tests will be conducted as control of the validated production process after release of the product according to the established procedures.

The final product will be formulated as sterile solution for IV injection. The radioactivity of the final product will be verified before injection using a suitable counter, as established on site.

Further details can be found in the Piramal Investigator's Brochure.

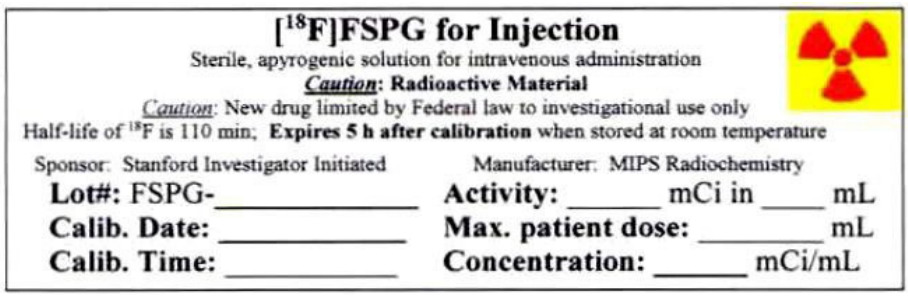
Dosage and Administration

Study participants will be administered ^{18}F-FSPG under the direct supervision of a nuclear physician or designee. For administration of ^{18}F-FSPG, access into a large vein (e.g., antecubital vein) should be established using a suitable indwelling catheter. To avoid extravasation of ^{18}F-FSPG, correct localization of the catheter must be ensured by a test injection of normal saline prior to the injection of ^{18}F-FSPG.

Each study participant will receive a single IV injection of ^{18}F-FSPG with a total activity amounting to 300 MBq (8.1 mCi) ± 10%. This single IV injection will contain a maximum mass dose of the diagnostic test agent (total: radioactive plus non-radioactive “cold” substance) of no more than 50 µg and a total volume of up to 10 mL. ^{18}F-FSPG will be administered manually via IV injection followed by a 10 mL saline flush. The PET/CT scan will be conducted about 60 minutes after the injection of ^{18}F-FSPG.

Packaging and Labeling

^{18}F-FSPG for Injection will be provided as a sterile solution in a glass vial, contained in a lead-shielded transport container. Provided is a representation of the ^{18}F-FSPG for Injection label to be used on both the vial and the lead-shielded transport container.



Imaging Agent Logistics and Accountability

The imaging agent will be prepared at Stanford at the following facility under the supervision of Director Frederick T Chin, PhD:

Cyclotron & Radiochemistry Facility
Lucas Building
Molecular Imaging Program at Stanford (MIPS)
1201 Welch Road, Room PS049
Stanford, CA 94305
P: 650-725-4182
F: 650-618-0415

The imaging agent will be requested via a standard online radiotracer request form received by the MIPS Cyclotron & Radiochemistry Facility at Stanford University. The agent will be hand delivered from the Radiochemistry Facility at the Lucas Center to the Hot Lab in the Nuclear Medicine and Molecular Imaging Clinic at Stanford Hospital.

Appropriate documentation (for order/receipt, dosage instructions and release for human use) will be provided with each delivery of imaging agent which will contain the batch number, time of preparation, and radioactive concentration of injection (MBq/mL) at the time of calibration preparation. The final quality control processes for the imaging agent will occur and be documented before the substance is administered to the subject at the imaging clinic. The qualified person at the manufacturing site will ensure that the product quality meets the defined criteria and will declare this in a document for release, to be received by the investigator (or designated personnel) at the imaging clinic. The imaging agent will not be administered to the subject without release for human use.

The investigator (or designated personnel) will confirm receipt of the imaging agent in writing and will use the imaging agent only within the framework of this clinical study and in accordance with this study protocol. For each subject he/she will keep a record of the imaging agent dispensed including the tear off label of the dispensed vial, a printout providing relevant parameters of synthesis and formulation of the imaging agent, and all other accompanying forms to the imaging agent. These documents are to be filed in the investigator site file.

Receipt, distribution, and return of the imaging agent must be properly documented on the forms provided by the manufacturer giving the following information: study protocol number, sender, receiver, date, mode of transport, quantity, batch number, expiration date, and retest date, if applicable.

5. STUDY PROCEDURES

Phlebotomy:

Blood will be collected once by a standard venipuncture. Blood will be collected in chemistry tubes (EDTA) and cell preservative tubes (Streck) to be stored in the Canary Center at Stanford biorepository and to be sent to our Boston University collaborators for processing and analysis. A total of 30 mL will be collected in a one-time blood draw. Blood draw will take place within 1 month after the patient has been consented.

Nasal Brushing:

Patient will be asked to blow their nose. A small speculum is used to widen the nostril and a tiny brush (the size of a Q tip) is put in through the speculum and rotated for 3 seconds. This brush is then removed, and a second brush is inserted and rotated for 3 seconds. Finally, the speculum and brush are removed from the nose. Nasal brushing will take place within 1 month after the patient has been consented.

^{18}F-FSPG PET/CT scan:

The patient will be injected with the radiotracer and allowed to rest for about 60 minutes. He/she will then be placed in the PET/CT and a static whole-body scan will be obtained from the vertex of the skull to the mid-thighs, starting at 60 (± 10) minutes after radiotracer injection.

6. STUDY CALENDAR

	Visit 1	Within 1 month from Visit 1 (can be Visit 2/3)	Visit 2 or 3*	Visit 3 or 2*	Within 24 to 72 hours after ^{18}F-FSPG PET/CT
Informed consent	X				
Nasal Brushing		X			
Blood draw		X			
^{18}F-FDG PET/CT			X		
^{18}F-FSPG PET/CT				X	
Patient follow-up					X

** & ↔ : PET/CT exams is not specified and may be reversed. PET/CT exams will be scheduled at least 24 hours apart, and no more than 2 weeks apart.*

7. COMPENSATION

Study participants will be compensated with a total of $500 for the time it takes them to complete the study procedures, including time and transportation for the additional visit to the clinic for the PET/CT procedure.

8. ADVERSE EVENTS AND REPORTING PROCEDURES

8.1. Risks and Potential Adverse Events

The risk associated with phlebotomy is small and includes discomfort, a bruise at the point where the blood is taken, redness, infection, and a rare risk of fainting. The risk associated

with the nasal brushing procedure is also small and includes minor discomfort and temporary nose bleed.

^{18}F-FSPG has been administered to over 65 cancer patients world-wide with no adverse events reported. As such, there are no major risks that are known or expected with this radiopharmaceutical. However, general risks to keep in mind include the radiation exposure. The expected radiation exposure of an ^{18}F-FSPG scan in this study is expected to be similar to or less than that of an ^{18}F-FDG scan. Other risks to patients are mainly related to the intravenous (IV) injection. Although the risk is small, intravenous injection can cause infection, injection site discomfort, and bruising.

8.2. Adverse Event Reporting

Patients will be contacted for follow up within 24 to 72hours after the ^{18}F-FSPG PET/CT, Adverse events will be graded according to CTCAE v5. Both Serious and Non-Serious Adverse Events will be clearly noted in source documentation. The Protocol Director (PD) or designee will assess each Adverse Event (AE) to determine whether it is unexpected according to the Informed Consent, Protocol Document, or Investigator's Brochures, and related to the investigation.

9. REGULATORY CONSIDERATIONS

9.1. Investigational New Drug Application (IND)

This study will be submitted to ^{18}F-FSPG IND 125516, held by Dr Andrei Iagaru.

9.2. Institutional Review of Protocol

The protocol, the proposed informed consent and all forms of participant information related to the study (e.g. advertisements used to recruit participants) will be reviewed and approved by the Stanford IRB and Scientific Review Committee (SRC). Any changes made to the protocol will be submitted as a modification and will be approved by the IRB and SRC prior to implementation. The Protocol Director will disseminate the protocol amendment information to all participating investigators.

Sample management

PET imaging analysis will be performed at Stanford. The blood and nasal samples will be transferred to Boston University for the RNA and DNA sequencing analyses.

9.3. Data Management Plan

We will use OnCore and REDCap for data storage and all paper documents will be stored in a binder in a locked cabinet on Stanford premises.

9.4. Data and Safety Monitoring Plan

During the clinical investigation, the Protocol Director will evaluate the progress of the trial, including periodic assessments of data quality and timeliness, participant recruitment, accrual and retention, participant risk versus benefit, and other factors that can affect study outcome. The study team meets bi-weekly to review the study conduct and status.

The Stanford Cancer Institute Data and Safety Monitoring Committee (DSMC) will audit study related activities at least annually in accordance with the DSMC SOP to determine

whether the study has been conducted in accordance with the protocol, local standard operating procedures, FDA regulations, and Good Clinical Practice (GCP). This may include review of regulatory binders, case report forms, eligibility checklists, and source documents. In addition, the DSMC will regularly review serious adverse events and protocol deviations associated with the research to ensure the protection of human subjects. Results of DSMC audits will be communicated to the IRB and the appropriate regulatory authorities at the time of continuing review, or in an expedited fashion, as needed.

10. MEASUREMENTS

10.1. Primary Outcome Measure (ClinicalTrials.gov)

The primary outcome is specificity of 18F-FSPG to discriminate between benign and malignant lung nodules, defined as the proportion of benign cases judged to be benign. With an expected 60 benign cases, an observed 90% specificity would have a 95% confidence interval of 79% to 96%. An observed specificity of 75% would have a confidence interval of 62-85%.

If a patient has multiple nodules, the per-patient assessment will be based on the dominant nodule only, defined as the largest nodule.

Outcome Title: ^{18}F-FSPG specificity for malignant lung nodules.

Outcome Description: ^{18}F-FSPG accumulation in lung nodules will be assessed. The observed FSPG specificity will be assessed against a fixed value of 75%.

Timeframe: Up to 5 years.

Safety Issue: No.

10.2. Secondary Outcome Measure (ClinicalTrials.gov)

The secondary outcome is improved performance of an integrated prediction model to discriminate between benign and malignant lung nodules, compared with a clinical prediction model.

Outcome Title: Improved prediction model for lung nodules

Outcome Description: Improved performance is defined as a $p < 0.05$ increase in the C statistic determined using DeLong's test for correlated ROC curves.

Timeframe: Up to 5 years.

Safety Issue: No.

10.3. Measurement Methods

For RNA and DNA sequencing the blood and nasal samples will be transferred to Boston University. PET imaging analysis will be performed at Stanford.

Total RNA-seq of Nasal Brushings: RNA sequencing libraries will be prepared from total RNA samples using Illumina® TruSeq® Stranded Total RNA Library Preparation Kit. The libraries from individual samples will be multiplexed in groups of 4 for cluster generation on

the Illumina® cBot® using Illumina® TruSeq® Paired-End Cluster Kit. Each sample will be sequenced on the Illumina® HiSeq® 2500 to generate paired-end 75 nucleotide reads. De-multiplexing and creation of FASTQ files will be performed using Illumina CASAVA software. RNA-Seq reads will be aligned to the human genome (hg19) using STAR, quality metrics will be calculated using RSeQC, and gene and transcript expression levels will be summarized using RSEM and Ensembl annotation. Gene and transcript filtering will be conducted on normalized counts using the mixture model in the SCAN.UPC R package. R software will be used to conduct all further analyses. Batch correction will be done using a modified version of our SCAN algorithm29 that is adapted to work with sequencing counts.

Single Cell RNA-seq of Nasal brushing: Nasal brushings will be subject to Florescent Activated Cell Sorting (FACS) (10 markers specific to either immune or epithelial cells) and sorted into single cells. Plates of single cells will be processed for single cell RNA sequencing using the CEL-Seq2 protocol, 75 bp single-end reads (400K/cell), on 200 single cells/sample.

Circulating tumor DNA: ctDNA lung cancer biomarker will be developed using plasma samples available on 380 patients (n = 190 each cases and controls). This biomarker will then be validated on the remaining 200 samples. We will analyze plasma samples using TEC-seq. Tumor WES sequencing will be performed on a subset of training set (n-150) to confirm source of ctDNA. Using the same genomic libraries utilized for TEC-seq, we will also perform whole genome analyses to identify chromosomal abnormalities using the Digital Karyotyping approach. TEC-seq and whole exome seq profile somatic mutations which is not considered genetic.

PET Imaging: Patients will be imaged by ^{18}F-FDG PET/CT and ^{18}F-FSPG PET/CT. Uncorrected maximum lesion intensity (SUV_{max}) along with qualitative measures (absent, minimal, moderate, or high uptake) will be recorded. These data will be analyzed for lung cancer diagnosis using our prospective cohort study design The person assessing SUVmax values and making the decisions about “positive” or “negative” will be blinded to the cancer status of the patient. We will maintain this by keeping the image reader blind to the patient information.

11. STATISTICAL CONSIDERATIONS

11.1. Statistical Design and Data Analysis

Clinical diagnosis is based on 1) histopathology of biopsy/surgical specimen or 2) surveillance by CT scans for a period of 2 years to confirm that there is no growth of the nodule. Meta-analysis shows that 18F-FDG has a sensitivity and specificity of 89% and 75% respectively (16). We hypothesize that 18F-FSPG is more specific. With an expected 90% specificity for diagnosis with 18F-FSPG, a sample size of 60 will have 90% power at an alpha-level of 0.05 to demonstrate a significant increase in specificity from a fixed value of 75%.

Integrated predictions models:

Our biomarker discovery and validation will use samples from 930 patients in the intended use population collected prior to diagnosis; and subsample from all available cancer and non-cancer patients. Through our experience with the NCI Early Detection Research Network (EDRN), we have a detailed plan for biomarker discovery and validation, which is briefly summarized here.

Identification of candidate genes from nasal total RNA-seq data: We will initially select 20 to 40 candidate genes that significantly discriminate between cases (n = 100) and controls (n = 100). We will prioritize candidates to: 1) represent cancer-associated gene co-expression patterns, 2) genes whose expression is independent of clinical factors, and genes in significantly enriched functional categories.

Using single cell nasal RNA-seq to refine the nasal gene expression biomarker assay: We hypothesize that a subset of airway cells harbor lung-cancer associated gene expression differences (or that the distribution of cell types might distinguish the airway of cancer patients). We will perform scRNA-seq on cells collected prospectively from 200 patients (n = 100 each cases and controls). We will identify cell subpopulations using Celda and test for cell type-specific alterations associated with subtype, prognosis or disease activity using a strategy similar to that used for bulk RNA-seq data. Additionally, we will test for differences in cell population frequency associated with prognosis or disease activity; and also determine which cell subpopulations express the prognosis and disease activity associated genes identified in the bulk RNA-seq to understand the cellular architecture of these disease signatures. For biomarker development, we will interrogate the cellular architecture of the expression of the nasal biomarker genes. If biomarker gene expression localizes to specific cells, we would subsequently explore whether enriching for these cells improves biomarker performance. Alternatively, if the frequency of a nasal cell type differs in patients with lung cancer, we would explore whether quantifying this cell-type's frequency could serve as a lung cancer biomarker.

Identification of CT features: Similar to the gene expression analysis, our initial goal is to identify 5 to 10 candidate semantic or quantitative imaging features that optimally discriminate cases and controls. We will centrally process thin-section CT images resulting in a high-dimensional dataset of image features based on CAD detection and nodule segmentation, and quantitative feature extraction. Semantic analysis of CT scans will be conducted by thoracic imagers using an illustrated lexicon to maximize inter-reader agreement. A convolutional neural network (CNN) will derive synthetic quantitative measures predictive of cancer status via cross validation. The most cancer-discriminating quantitative and semantic features will be selected for downstream analysis.

Data Integration: Using a new set of 150 patients (75 cases and 75 controls), we will subset candidate genes and imaging features based on whether they improve (significantly higher ROC curve AUC) clinical lung cancer risk models with the goal of picking no more than 10 genes and 5 imaging features, and prioritizing genes least correlated with imaging features or the ctDNA biomarker. We will next use data from 380 new patients (n = 190 each cases and controls) to train and "lock down" models prior to validation. Model building will use a logistic regression framework (incorporating LASSO and ridge regression) and cross-validation. We will build models stepwise from clinical-variable-only models by adding additional biomarker datatypes (gene expression, imaging, and / or the ctDNA biomarker).

Cross validation will determine the gain in diagnostic performance from each additional datatype, and combinations that do not improve performance will be eliminated. These locked down models will be validated in the remaining samples (n = 200). The primary endpoint will be calculated after every patient has either completed two years of follow up, been diagnosed positive, or died, whichever comes first. The primary endpoint for validation of this prediction model will be replication of improved performance. Exploratory

endpoints will determine the range of pre-test probabilities for which the models have potential clinical utility.

11.2. Sample Size

Primary outcome: To compare 18F-FSPG PET/CT imaging with 18F-FDG PET/CT imaging for indeterminate lung nodules we will evaluate 120 patients. We estimate that approximately 60 of the patients will be diagnosed with lung cancer after complete diagnostic work-up and ~60 will not have lung cancer. Meta-analysis shows that 18F-FDG has a sensitivity and specificity of 89% and 75% respectively (16). We hypothesize that 18F-FSPG is more specific. With an expected 90% specificity for diagnosis with 18F-FSPG, a sample size of 60 will have 90% power at an alpha-level of 0.05 to demonstrate a significant increase in specificity from a fixed value of 75%.

Secondary outcome: In total the SU2C consortium cohort will be comprised of 930 study participants. Our Stanford group will contribute 120 (60 cases and 60 controls) to the larger sample set. Breakdown of the 930 patients are as follows. For the discovery step, sample size of 100 cases and 100 controls provides 80% power to detect RNA markers with effect sizes of at least 0.52 assuming that we will control the false discovery rate at 10% and 5% of markers are differentially expressed. For the refinement step, 75 cases and 75 controls provides 81% power to detect markers with a ROC AUC of at least 0.63 assuming an alpha level of 0.05. No correction for the multiple hypothesis testing is planned as we will re-evaluate top candidates. The sample size of 190 of each group for model building will support the following:

1. This sample size will be sufficient to include approximately 12 to 20 variables overall between clinical, imaging, ctDNA and gene expression markers based on conventional rules of thumb of 10 to 15 subjects per group per variable;
2. This will allow us to estimate the AUC for each model with a precision of approximately 4.5% (95% CI);
3. Provide 80% power for detecting delta AUCs between models of 0.06 assuming model correlation of 0.6.

For the model testing, 100 subjects in each group will provide AUC precision of ± 0.06 (and a precision of between 0.06 and 0.08 for the sensitivity and specificity), as well as a 80% power to detect differences in AUCs between models of 0.09. IRBs at the other sites have approved the collection of biospecimens and use of clinical / imaging data for this model development and validation work.

11.3. Accrual estimates

In our clinic on average 30 patients per month are seen with lung nodules. Of these patients around 50% have truly indeterminate lung nodules for which clinical management is uncertain. We estimate that about 20% of the eligible candidates will consent to participate in the study, leading to an accrual of circa 3 patients per month. We expect to be able to complete the study (50 patients) within 3 years.

After the first 2.5 years of the study, we will assess the long-term follow up rate and add

additional patients if accrual is not sufficient. We will not do an interim statistical analysis.

11.4. Criteria for future studies

If ^{18}F-FSPG PET/CT imaging has a higher specificity for detecting malignant lung nodules compared to ^{18}F-FDG PET/CT, a larger study will be planned.

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