A Pilot Study of Exhaled Breath Analysis to Predict Risk of Symptomatic Pneumonitis After Chemoradiotherapy For Stage III Non-Small Cell Lung Cancer
Wake Forest Baptist Comprehensive Cancer Center (WFBCCC)
WFBCCC 98119

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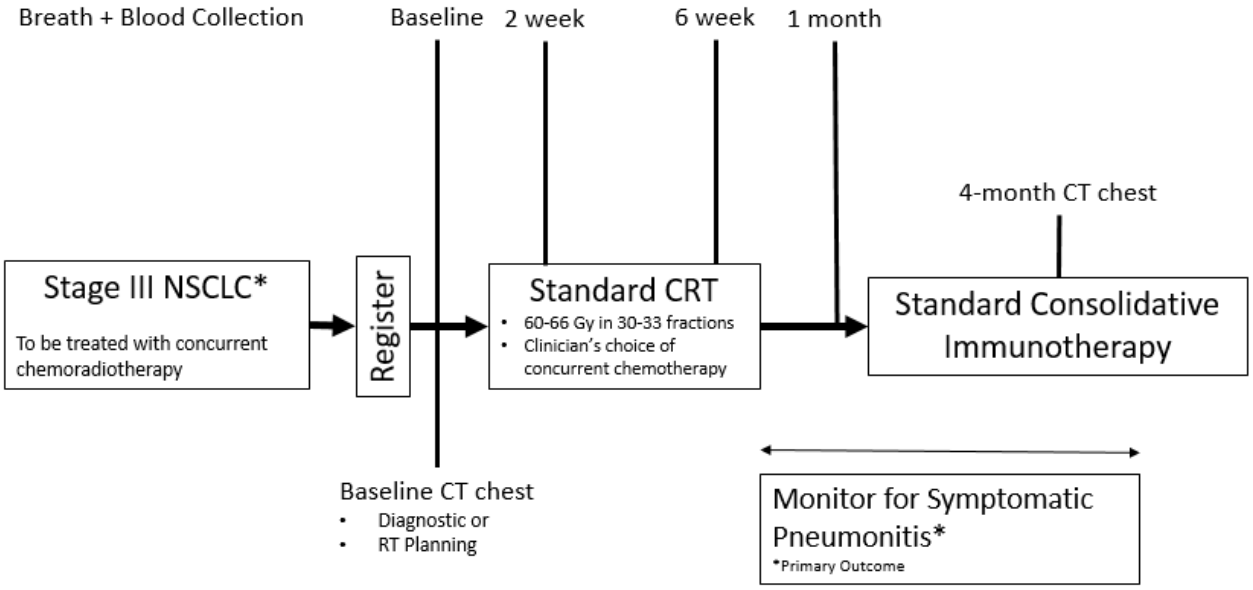
Study Schema



1.0 Introduction and Background

Summary of Pneumonitis in Patient with Stage III NSCLC

Definitive chemoradiotherapy (CRT) followed by consolidative immunotherapy is a standard treatment for locally advanced non-small cell lung cancer (NSCLC) (1, 2). Pneumonitis represents injury to the lungs in the form of acute or chronic inflammation as a result from these treatments. This injury can either be asymptomatic or symptomatic, and may cause cough, shortness of breath, oxygen requirement, or worse. In patients treated with CRT followed by standard of care immunotherapy, symptomatic pneumonitis (SP), defined as Common Toxicity Criteria for Adverse Events (CTCAE) grade 2 or higher pneumonitis, occurs in approximately 15-20% of patients (3, 4). Current treatment of SP is largely dependent on symptom severity. This ranges from observation or anti-tussive medications for mildly symptomatic cases, to prolonged corticosteroid use, oxygen supplementation, or even hospitalization in more severe cases. Rarely, death may result from SP. The most prevalent etiologies of pneumonitis in these patients includes radiation pneumonitis and immune-mediated pneumonitis. Radiation-induced lung injury, which includes radiation pneumonitis and pulmonary fibrosis, occurs with variable severity (from asymptomatic to fatal) in 20% to 50% of patients treated with modern CRT (5, 6). Radiation pneumonitis generally refers to a reversible, subacute inflammatory reaction that occurs within several weeks to months of CRT (7). Immune-mediated pneumonitis, exhibited by focal or diffuse inflammation of the lung parenchyma, occurs in approximately 5% of patients receiving immune checkpoint inhibitors (8).

Currently, it is very difficult to identify patients at high risk of SP prior to treatment and limited data exist to support individualized prophylactic strategies for those at highest-risk of SP (9). Given the relatively sparse tools at hand to predict an individual's risk of developing SP after combined-modality therapy for locally advanced NSCLC, there is a dire need for more sensitive, personalized methods of detecting patients at risk. The main technique presently available to reduce an individual's risk of SP is to limit the radiation dose to normal lung tissue. Prior studies discussed below have demonstrated correlations between serum markers, but these findings have not been routinely employed for risk stratification in current practice.

Personalized Strategies to Mitigate Pneumonitis Risk Are Lacking

There are guidelines in place for minimizing radiation dose to normal lung tissue with the goal of decreasing the risks of pneumonitis (10). However, the most appropriate dosimetric thresholds are controversial and are rather crude in their ability to predict SP after radiotherapy. Current practice is to apply generic lung constraints to all patients receiving CRT, generally ensuring that the amount of bilateral normal lung tissue receiving at least 20 Gy is less than 30-35%. Such constraints were devised prior to common use of immunotherapy in this patient population. Given that immunotherapy

alone can induce symptomatic pneumonitis, it is unclear if these constraints remain appropriate. Development of biomarker-based risk prediction models of SP may facilitate further investigation of customized prophylactic strategies (i.e. inhaled corticosteroids or short courses of systemic steroids during CRT).

Serum biomarkers including transforming growth factor-beta 1 (TGF-β1), interleukin (IL)-6, IL-1α, and IL-10 have been associated with development of SP (11-14). Previous studies have combined both dosimetric and serum TGF- β1 to estimate the risk of SP (15). However, inconsistent reports and the use of frequent blood draws during CRT has made this approach difficult for patients and clinicians to implement in practice. Additionally, these studies were performed prior to the adoption of consolidative immunotherapy after CRT. Due to these limitations and inconsistencies, better predictive metrics, collected non-invasively, are needed to identify patients at high risk for SP who may benefit from personalized radiation therapy or prophylactic medical therapy.

Because of the inconsistencies described above, we plan to include serum analyses (Collected during routine labs) for each of the target biomarkers that will be analyzed in exhaled breath samples. Serum and exhaled breath are two different sources of biological sampling and will provide different metabolic information which may be useful as we elucidate the association between our targeted biomarkers and SP in the era of immunotherapy. Serum biomarkers can be physiologic references but given the proximity of the breath analyses to the localization of injury, breath biomarkers may provide a clearer window in which to correlate predictive markers with SP development.

<u>Exhaled Breath Analyses for Risk Stratification</u>

Exhaled Breath Condensate (EBC) and Exhaled Breath Volatiles (EBV) can be non-invasively captured by collecting air as the patient exhales over the course of 5-10 minutes into a commercially available R-tube and ReCIVA device, respectively (Figure 1a and 1b). These collections provide biological samples that are a rich source of metabolic and genetic information and have the potential to result in novel biomarkers predictive of SP. Prior studies have detected various cytokines, proteins, and other molecules in the EBC of lung cancer patients and TGF-β1 in EBC of patients with sarcoidosis (16). Exhaled octane has the potential to predict development of acute respiratory distress syndrome (17). These findings suggest that we will be able to identify these and potentially other molecules in our cohort of NSCLC patients to correlate with SP. One previous study has demonstrated the feasibility of using exhaled breath analyses to predict radiation pneumonitis in patients treated

Figure 1b: EBV collection device

Figure 1a: EBC collection device

with stereotactic body radiotherapy (18), but this has not been attempted in locally advanced lung cancer patients receiving CRT.

EBV analyses as described have not been performed using unbiased metabolomics. A metabolomics approach is able to obtain a snapshot of biological and molecular mechanisms underlying a disease process by capturing metabolic profiles that are indicative of cellular metabolism. Current profiling technology results in the identification of hundreds to thousands of metabolites, which can be used as an unbiased analysis of biochemical pathways and potentially target biological dysfunction (19-21).

Recent studies have also focused on the link between cancer and the human microbiota, with special interest in determining how the microbiota contributes to the pathogenesis of various cancer types, including lung cancer (22). Similarly, other studies have illustrated that alterations in the rodent gut microbiome are capable of potentiating radiation-induced bowel injury (23). Mechanistically, the gut microbiome is thought to directly impact gastrointestinal immune cells (24). It is well-established that the lungs are not sterile, and scientists have recently demonstrated that the lung microbiome is very similar to the oral microbiome (25-27). An informative approach to assessing the human microbiota is through sequencing, which allows the characterization of microorganisms and provides information on the putative function of the microbiota. Microbial 16S rRNA is also detected in exhaled breath condensate (EBC), but its impact regarding SP has not been explored (28). Collecting EBC before CRT offers a noninvasive approach for studying the role the normal pre-treatment microbiota plays in SP, which has not previously been performed.

1.1 Summary and Rationale

In this proposed pilot study, we seek to measure exhaled breath-derived biomarkers at different points in the CRT timeline (baseline, at 2 weeks, 6 weeks, and 1 month post-treatment). We hypothesize that these measurements will have measurable differences between each time point, and, further, that differences relative to baseline will be associated with the development of SP in a group (n=50) of stage III NSCLC patients treated with CRT followed by immunotherapy. As part of this goal, we will also test the same biomarkers in serum to evaluate the association between serum and breath-derived biomarker concentrations and to determine the association between serum measurements and SP. We also will evaluate whether constituents of the lung microbiome prior to CRT are related to the development of SP.

2.0 Objectives

This is a prospective pilot study investigating exhaled breath condensate analyses to quantify the variability over time of various biomarkers associated with SP. Compounds of interest have been chosen due to previous studies demonstrating association with pneumonitis in analyses of serum. Our primary hypothesis is that biomarker concentrations will change over the course of treatment (pre-treatment,

2-weeks on treatment, 6-weeks on treatment, and at 1-month post-treatment, Section 2.1.1). Our secondary hypotheses are that these changes in biomarker concentrations will correlate with SP (Section 2.2.1), which we expect to occur in 15-20% of our patient sample, and that these changes will also occur in serum, which will also correlate with SP (Section 2.2.2). Finally, we hypothesize that pre-treatment EBC-derived microbial signatures will be different in patients who develop SP versus those who do not (Section 2.2.3).

2.1 Primary Objective

2.1.1 To quantify the intra-person variability of concentrations of TGF-β1, IL-6, IL-1α, and IL-10 measured in exhaled breath condensate.

2.2 Secondary Objectives

2.2.1 To examine the associations between differences in pre-treatment and post-treatment *EBC* concentrations of TGF-β1, IL-6, IL-1α, and IL-10 and the development of CTCAE grade 2+ SP.

2.2.2 To examine the associations between *serum* measures of TGF-β1, IL-6, IL-1α, and IL-10 and:

2.2.2.1 *EBC* measures of the same biomarkers, and
2.2.2.2 The development of CTCAE grade 2+ SP.

2.2.3 To examine the association between microbiome signatures found in pre-treatment EBC and the development of CTCAE grade 2+ SP.

2.3 Exploratory Objective

2.3.1 To examine the association between pre- and post-treatment exhaled breath volatile-based metabolites and the development of SP

3.0 Study Population

Patients with biopsy-proven non-small cell lung cancer who are planned to undergo standard of care treatment will be enrolled in this study. Treatment will include definitive chemoradiotherapy followed by consolidative immunotherapy. Patients who are not able or unwilling to provide breath samples will not be enrolled. Patients treated with any prior radiotherapy directed at the chest, or those taking corticosteroids at baseline will be excluded.

3.1 Inclusion Criteria

3.1.1 Age greater than or equal to 18 years old.

3.1.2 Clinically diagnosed or suspected Stage III non-small cell lung cancer to be treated with chemoradiotherapy as part of cancer treatment, as determined by the treating clinician

3.1.3 Plan for treatment with definitive radiotherapy (≥60 Gy) with concurrent chemotherapy at the discretion of the treating radiation and medical oncologists.

3.1.4 Willing and able to tolerate exhaled breath collection.

3.1.5 Able to provide informed consent.

3.2 Exclusion Criteria

3.2.1 Systemic (oral, intravenous or intramuscular) corticosteroid use for any reason within 5 days of registration.

3.2.2 Prior radiotherapy directed at the chest (thoracic inlet superiorly to diaphragm inferiorly).

3.2.3 Any systemic antibiotic use within 2 weeks of registration.

3.3 Inclusion of Women and minorities

Men and women of all races and ethnicities who meet the above-described eligibility criteria are eligible to participate in this study. The study consent form will also be provided in Spanish for Spanish-speaking participants.

Based on WFBCCC population estimates, we may expect approximately 44% of participants to be women. Translating this to our sample size estimate of 50 patients, we may enroll approximately 22 women. We may enroll approximately 10-13% Black or African American patients. Based on our catchment area and hospital demographics we do not expect high accruals of individuals of Hispanic/ Latino, American Indian/Alaska Native or Asian ancestry; however, no individual will be excluded from the study if they satisfy the above inclusion/exclusion criteria. Should we not meet or exceed these estimates, the PI will engage the Office of Cancer Health Equity to discuss strategies to enhance recruitment in these target populations.

4.0 Methods

4.1 Registration Procedures

All patients entered on any WFBCCC trial, whether treatment, companion, or cancer control trial, **must** be linked with a study protocol in EPIC within 24 hours of Informed Consent. Patients **must** be registered prior to the initiation of the study.

You must perform the following steps in order to ensure prompt registration of your patient:

1.0 Complete the Eligibility Checklist (Appendix A)
2.0 Complete the Protocol Registration Form (Appendix B)
3.0 Alert the Cancer Center registrar by phone, *and then* send the signed Informed Consent Form, Eligibility Checklist and Protocol Registration Form to the registrar, either by fax or e-mail.

Contact Information:
Protocol Registrar PHONE (336) 713-6767
Protocol Registrar FAX (336) 713-6772
Protocol Registrar E-MAIL (registra@wakehealth.edu)

*Protocol Registration is open from 8:30 AM - 4:00 PM, Monday-Friday.

4.0 Fax/e-mail ALL eligibility source documents with registration. Patients **will not** be registered without all required supporting documents.

Note: If labs were performed at an outside institution, provide a printout of the results. Ensure that the most recent lab values are sent.

To complete the registration process, the Registrar will:

- assign a patient study number
- register the patient on the study

4.2 Data Collection Methods

4.2.1 Clinical Data Collection

Implementation of this prospective observational pilot study has the full support of the Wake Forest Thoracic Multidisciplinary Research Committee. We plan to enroll 50 patients with newly diagnosed locally advanced non-

small cell lung cancer undergoing standard of care chemoradiotherapy. Baseline characteristics including demographics (age, sex, ethnicity, and race), tobacco use history, past medical history (COPD, interstitial lung disease, asthma, etc.), antibiotic and steroid use, and oncologic history (TNM stage, tumor size, location, histology and date of diagnosis) will be recorded. Baseline pulmonary function, tested as a part of routine pre-treatment workup, will be collected if available. Treatment-related factors will be recorded including radiotherapeutic modality (3D conformal radiotherapy, intensity-modulated radiotherapy, or volumetric modulated radiotherapy), detailed volumetric and dosimetric parameters for each patient's individualized radiation plan, as well as type of chemotherapy and immunotherapy used.

4.2.2 Exhaled Breath Sample Collection

At the baseline visit prior to the initiation of CRT, pre-treatment sample collection will occur within the Radiation Oncology Clinic. This may occur on the day of the radiation treatment planning CT simulation scan. Patients will be brought to a clinic room or a holding area depending on room availability. Each of these areas has access to medical air valves located on the wall. (Figure 2)

For exhaled breath condensate, we will utilize the R-tube collection device (Respiratory Research Inc., Figure 3). The R-tube is a one-time use apparatus that utilizes a metal cooling sleeve, placed over the plastic tube, to cool the patient's breath, creating condensation build up within the device. The patient will breathe normally through the mouth piece for 10 minutes. Following the collection, a metal plunger will be used to consolidate the condensate within the collection tube and the EBC sample can be transferred to a fresh sterile tube. All biofluids will be aliquoted, frozen and stored at -80° C until analysis.

After the initial exhaled breath condensate sample is obtained, the patient will be invited to provide a second breath sample for exhaled breath volatiles. For this, we will utilize the ReCIVA (Figure 1b, Owlstone medical), breath collection device. The device will be attached to the in-house medical air supply (yellow) with airflow set to 40L/min per the company's recommendation (Figure 2). This medical air is between 19-22% oxygen (similar to room air) and is readily available in the Radiation Oncology clinic rooms/holding area where breath samples will be obtained (Figure 2). One-time use masks will be placed over the patient's mouth and nose. The patient will be allowed to breathe normally to acclimate themselves to the mask and airflow. The patient will be allowed to hold the device, if desired, and a head strap will be placed around their head for additional support and comfort. Once the patient is acclimated (<1 min), they will be prompted to start the collection. Pre-loaded collection parameters will be set with the

Figure 2: Clinic breath collection setup

Figure 3: R-tube collection

device software to simultaneously collect four tubes of exhaled breath from patient's expired breath. Each collection tube (pictured in Figure 1b) is packed with material to bind volatile chemicals. Four (4) tubes constitute one sample at each time point. Once collection begins, the system will learn the breathing pattern of the patient (25 sec) and will adjust the collection pumps accordingly. Breath collection will take between 5-7 min, depending on the patient's breathing rate and total collection volume. This study will collection 500 mL of exhaled breath on each of the four tubes. All exhaled breath samples will be stored at 4°C until analysis. Due to the nature of the EBV, processing and analysis will occur in batches every 1-2 weeks. This essentially uses the entirety of the sample; the EBV samples will be spent after completion.

4.2.3 Blood Collection

Patients treated with CRT have blood counts checked prior to CRT and weekly during CRT to ensure their eligibility for concurrent chemotherapy.

This is part of routine standard of care in medical oncology. Additionally, routine blood count testing is performed approximately 1 month after completion of CRT, to ensure eligibility for consolidative immunotherapy as per the standard of care. In order to minimize the number of blood draws the patients are subjected to, we will plan to obtain research blood samples simultaneously with clinical blood draws at baseline (visit prior to CRT which will occur at the time of the RT planning CT simulation), the last blood draw within 1 week of the last fraction of RT, and at follow-up approximately 1 month after completion of CRT. With the patient's consent, we will obtain 1 x purple-top vacutainer EDTA tube and 1 x red-top tube in conjunction with these standard blood draws.

All samples collected from this study will be banked and will have the potential to be used for future scientific experimentation, which may include additional genetic, proteomic, and metabolomic analyses not described in the data collection section below.

4.2.4 Follow-up

Patients will be followed clinically and with routine CT imaging at baseline, 1 month, and 3-4 months post-treatment. Appointments will take place in the morning in Dr. Farris' clinic in the Radiation Oncology Department, 1st Floor Cancer Center. Upon entering the clinic, they will be taken to a room where the breath samples will be obtained as described above. Blood samples at follow-up will coincide with follow-up blood draws as part of routine care as described above. All samples collected from this study will be banked and will have the potential to be used for future scientific experimentation, which may include additional genetic, proteomic, and metabolomic analyses not described in the data collection section below. After four months of follow-up, the study will end and the patients will no longer be followed.

4.3 Research Data Collection and Analysis

4.3.1 Breath and Blood Biomarker Concentration Analysis

State-of-the-art AlphaLISA immunoassay kits (Perkin Elmer, Waltham, MA) will be used to measure the concentration of TGF-β1, IL-6, IL-1a and IL-10 in EBC and serum at baseline, 2-week after CRT start, 6-week after CRT start (end of RT) and 1-month post-CRT end. The AlphaLISA platform is a no wash immunoassay which has increased sensitivity and selectivity compared to the conventional ELISA assay. Assays will be analyzed on an EnVision 2105 Multimode plate reader (Perkin Elmer, Waltham, MA). Measurements for known standards and samples at each time point will be

performed in triplicate. Concentration curves will be plotted in Excel (Microsoft) and used to determine sample concentrations.

4.3.2 Breath Microbiome Analysis

DNA will be extracted from each EBC sample using the QIAamp® UCP Pathogen Mini Kit (Qiagen) with extended homogenization of the breath samples using the Mini-Beadbeater-96 cell disrupter (Biospec Products). The quantity and quality of the DNA extracts will be determined using a DeNovix DS-11 FX+ Spectrophotometer/Fluorometer and Agilent 4200 TapeStation, respectively. The DNA will then be prepared for shotgun metagenomic sequencing using the Nextera® DNA Flex Library Preparation Kit (Illumina). DNA libraries will be normalized and quantified prior to pooling to ensure equal library representation. Pooled DNA libraries will then be sequenced using the Illumina NovaSeq 6000 S1 Reagent Kit and sequencer, generating 2 x 150 bp paired-end reads. Raw sequence reads (FASTQ files) will be imported into the Microbial Genomics Pro Suite within the CLC Genomics Workbench (version 11.0.1; https://www.qiagenbioinformatics.com) for QC, adapter trimming, and analysis. High-quality (≥Q30) reads for each sample will be aligned to those in a blank extraction sample (negative control) that will be processed from DNA extraction through library preparation to identify and remove contaminating sequences. The filtered reads will then be aligned to a reference database consisting of complete genomes in the RefSeq database for prokaryotes (bacteria and archaea), eukaryotes (fungi and protozoa) and viruses for taxonomic profiling. The filtered reads will also be assembled into contigs *de novo*, and MetaGeneMark (29) will be used to identify protein-coding regions within the reads to predict functional diversity of the microbiota identified in the samples. The coding regions will be annotated with Pfam domains and GO terms, and the abundance of functional categories will be estimated to build a functional profile for each sample.

The results from the taxonomic and functional profiling will each be merged to estimate similarities and differences among the samples. Heat maps will be generated using Euclidean distance and clustering based on average linkage to assess functional similarities between samples, and principal component analysis (PCA) ordination of Bray-Curtis similarity between samples will be used to visualize the microbiota diversity across the samples.

4.3.3 Exhaled Breath Volatile Data Generation and Metabolomic Analysis

Unbiased metabolomics analysis of bio-volatiles and biofluids has the potential to identify 100-1000’s of metabolites in any single sample. Our

group has successfully assessed bio-volatiles and biofluids by unbiased metabolomics (30, 31). Although there is no single method that allows for the complete analysis of the metabolite, utilizing multiple modes of sample preparation and analysis will potentially identify a number of classes of compounds relevant to this study. The bio-volatile that will potentially be identified are various hydrocarbons, aldehydes, ketones, and alcohols.

Exhaled breath samples will be analyzed using a Markes thermal desorber, TD100-xr, to inject breath samples onto an Agilent two-dimensional GC system 7890B in tandem with LECO's Pegasus 4D Time-of-Flight instrument system (TD-GCxGC-TOFMS). The collected spectra will be processed using LECO's ChromaTOF version 4.72 software. Initial data processing includes peak deconvolution, 1st and 2nd dimension retention time recordings, signal to noise ratio calculation, unique mass detection, peak area determination, and compound identification based on the spectral pattern similarity match against established mass spectral reference libraries (NIST17, 2017 National Institute of Standard Technology, NIH, USA). Identified spectral matches will be aligned using R-program R2DGC (32) to determine common metabolites in patients prior to and after CRT. MetaboAnalyst 4.0 will be employed to normalize and transform the data for univariate and multivariate statistical analysis (33). Additional statistical analysis may be performed using Graphpad Prism 8 (Graphpad Software, Inc).

4.3.4 Data Storage

All research data collected on this study will be stored on Wake Forest Baptist Medical Center password protected computers and servers.

5.0 Study Outcomes and Outcome Measures

5.1 Primary Outcome

5.1.1 Concentrations of TGF-β1, IL-6, IL-1α, and IL-10 (ng/mL) measured in exhaled breath condensate prior to CRT (baseline), at 2 weeks after CRT start, 6-weeks after CRT start (the end of CRT), and 1 month after completion of CRT.

5.2 Secondary Outcomes

5.2.1 Concentrations of TGF-β1, IL-6, IL-1α, and IL-10 (ng/mL) measured in exhaled breath condensate prior to CRT (baseline), at 2 weeks after CRT start, 6-weeks after CRT start (the end of CRT), and 1

month after completion of CRT compared among patients who develop SP versus those who do not. SP will be assessed at each time point and will be defined as presence of CTCAE version 5.0 grade 2 or higher SP (Appendix C).

5.2.2 Concentration of TGF-β1, IL-6, IL-1α, and IL-10 (ng/mL) measured in serum prior to CRT (baseline), at 2 weeks after CRT start, 6-weeks after CRT start (the end of CRT), and 1 month after completion of CRT:

5.2.2.1 Compared to concentrations of same biomarkers measured in exhaled breath condensate at same time points (see 5.2.1);
5.2.2.2 and compared among patients who develop SP versus those who do not.

5.2.3 Microbial signatures measured in exhaled breath condensate prior to CRT, and presence/absence of SP at study end.

5.3 Exploratory Outcome

5.3.1 Metabolomic signatures associated with the development of SP. This will include quantitative assessments of metabolic characteristics measured at prior to CRT (baseline), at 2 weeks after CRT start, 6-weeks after CRT start (the end of CRT), and 1 month after completion of CRT. Quantitative assessments of metabolomic assessments measured prior to CRT. Using these three sets of quantitative assessments (at each of the three time points), “signatures” will be developed (described in statistical analysis section) and compared to see whether there are characteristics that remain similar or change when comparing SP and no-SP measures.

6.0 Analytic Plan

6.1 Analysis of Primary Outcome, Sample Size, and Power

This is a pilot study; therefore, our objectives are exploratory in nature, and we have little to no pre-existing findings reported in the literature to guide our expectations. With this in mind, we plan to recruit a single cohort of 50 patients. In order to be able to examine the questions concerning intra-person variability in biomarker measures over time, and the possible relationships of breath and microbial biomarker signatures with lung cancer outcomes, both pre- and post-radiation treatment measures are needed on each participant. Our goal is to

collect from each patient one pre- (T0) and two post-treatment (T1, immediately post treatment and T2, one month following completion of treatment) samples of breath and serum to be examined. Our target sample size of 50 patients is the target for patients with pre- and post- samples collected. Our main interest is in differences between biomarker levels at T1-T0, where we expect to see the largest differences.

First, for the primary objective of detecting intra-person variability from T1 to T0 in EBC measures, the table below shows different power levels for n=50 patients, using a 2-tailed alpha of 0.05.

Detectable standard deviation units	Power
0.3	0.55
0.4	0.79
0.5	0.93

6.2 Analysis of Secondary Outcomes

To examine the association between differences in pre-treatment and post-treatment EBC concentrations of biomarkers and development of SP, we will conduct 2-group independent t-tests comparing mean change levels (from T0 to T1) in each marker among those who developed SP (expected to be around 10-15 patients) to those who remained free of SP at the study end. We will also conduct various exploratory logistic regression models, if numbers permit, to predict the outcome of SP, using various biomarker values (e.g., T0, difference between T0 and T1, etc.) as key predictors. If numbers permit, we will also examine results from repeated measures logistic regression models, using the three repeated measures of the biomarkers as predictors, and/or using at least one delta score as a predictor. Our goal in such exploratory modeling will be to discern what time points or what differences in EBC biomarkers appear to be most highly predictive of occurrence of SP. We will conduct most of this modeling on a single biomarker at a time; based on biological rationale, bivariate findings, and numbers, we may also explore some modeling where more than one biomarker is included.

We also wish to determine to what degree serum levels of biomarkers correlate with levels of the same biomarkers measured in exhaled breath condensate, and how well these serum markers predict SP. For this latter goal, we will use a similar plan as described in the preceding paragraph.

For the former objective (examining how well serum and breath levels of biomarkers correspond), because we do not hypothesize a priori that these 2 different sources of a biomarker are actually measuring (estimating) the same quantity for any of the markers, we do not expect absolute agreement. We will

thus first examine scatterplots of the serum vs breath measure of each biomarker, and if the association appears monotonic in the scatterplot, we will compute Spearman rank correlations. (If the association appears linear and monotonic, we will compute Pearson correlations.) If an association between a serum and breath measure of a biomarker is non-monotonic, we will attempt to fit a curve describing the association most accurately.

For our final secondary objective (examining the association between microbiome signatures found in pre-treatment EBC and development of SP) we will use cluster analysis methods to determine whether SP and non-SP patients share similar marker profiles. As stated above, this is a pilot study, therefore these cluster analyses will be considered exploratory and hypothesis generating.

6.3 Analysis of Exploratory Outcome

For the analysis of metabolic measures, we will examine the association of metabolic signatures with development of SP. We hypothesize that metabolic markers in patients who develop SP will be different than those who do not. Therefore, a first analysis will be to examine a series of paired t-tests to determine which markers significantly change between the two time points for patients.

In addition to this analysis, we wish to determine whether there are any marker signatures that appear in groups of patients. To do this we will use cluster analysis methods to determine whether there are any groups of patients who share similar marker profiles at baseline and post-CRT, or change from pre- to post- for metabolic measures. If we are able to identify clusters of patients with common marker signatures, we will then determine whether there are other disease characteristics that are shared by these patients (i.e., tumor stage, lung function, etc.).

6.4 Accrual Rate

Due to the large volume of NSCLC patients treated at this institution, we generally see 4-5 patients per month that would meet inclusion criteria. Considering those that meet eligibility but do not enroll, we expect an accrual of approximately 2 patients per month.

6.5 Length of Study

In order to accrue 50 patients at a rate specified above, the length of the accrual will be approximately 2 years. The study will complete after the 4-month follow-up of the last patient and we estimate the total duration at no longer than 2.5 years.

7.0 Treatment Plan

7.1 Treatment Administration

This study is observational in nature and does not impact the treatment received by enrolled patients in any way. Patients will be treated according to the standard of care for stage III NSCLC. They will be seen in consultation by a radiation oncologist. When the decision to proceed with definitive CRT is made, patients will sign informed consent for their radiation treatment. They will also sign informed consent for concurrent chemotherapy at the discretion of the medical oncologist.

Treatment planning will comprise a CT simulation with or without contrast using appropriate immobilization at the discretion of the treating radiation oncologist. Methods to account for respiratory motion that may be utilized include 4-dimensional CT imaging and slow CT scan techniques. Target and normal tissue delineation as well as treatment planning will be performed at the discretion of the treating radiation oncologist. Specific radiotherapy dose/fractionation are not mandated by the protocol, but 60-66 Gy in 1.8-2 Gy daily fractions is recommended. Weekly on-treatment visits will be performed according to the standard of care.

Concurrent chemotherapy may be delivered at the discretion of the treating medical oncologist. There is not a mandated time frame for administration of concurrent chemotherapy with regard to the first fraction of radiotherapy, but it is recommended that first dose of concurrent chemotherapy occur within 72 hours of the first radiotherapy fraction – ideally on the same day, if possible.

7.2 Follow-up

After completion of CRT, patients will be followed with routine clinical visits and radiographic examinations according to the standard of care. This observational study utilizes clinical and CT imaging surveillance obtained as part of routine follow-up as outcome measures for SP. Patients will return for post-treatment follow-up visit at 1-month post-treatment for clinical assessment. Serum/EBC samples will be collected at this visit. Three months thereafter, at approximately 4 months post-CRT, a routine surveillance CT examination of the chest (with or without contrast) will be performed. Clinical assessment/follow-up will be performed at this time point as well, and the final sample collection will occur at this visit.

7.3 Study Calendar

	Prior to CRT Start	2-week after CRT Start[e]	6-week after CRT Start[e]	1 Month after CRT End[e]	4 Months after CRT End[e]
Informed consent	X				
Demographics	X				
Medical history (Appendix E)	X				
Physical Exam	X				
Pulmonary Function Tests (Appendix H)	X[i]				
Antibiotic and Steroid Use (Appendix D)	X	X[e]	X[e]	X[g]	X[g]
Smoking Status (Appendix G)	X	X[e]	X[e]	X[g]	X[g]
Breath Collection (EBC[a]/EBV[b,h])	X[d]	X[e]	X[e]	X[g]	
Blood collection[c]	X[d]	X[e]	X[e]	X[g]	
CT chest with or without contrast	X				X[g]
Pneumonitis Evaluation (Appendix C)	X			X[g]	X[g]
Dietary Evaluation (Appendix M)	X[d]	X[e]	X[e]	X[g]	
Radiotherapy Detail Form (Appendix I)	X[f]				

[a] Approximately 1-1.5 mL of EBC will be generated in 10 min of breathing
[b] 500 ml of EBV in each of 4 collection tubes will be collected in 5-7 min of breathing
[c] Blood sample in each redtop (x1) and purpletop (x1) vacutainer blood collection tube
[d] Pre-CRT sample collections to occur within 14 days of the date of RT start. It is recommended but not required that this occurs on the date of CT simulation.
[e] May occur within +/- 7 days of the scheduled time point measured from the date of RT start. The 2-week after CRT start, 6-week after CRT start, and 1-month after CRT time points will be coordinated with routine blood draws through medical oncology by the study research nurse.
[f] To be completed after completion of treatment planning. This may also be completed at any time after CRT has started.
[g] May occur within +/- 14 days of the scheduled time point measured from the date of RT end.
[h] EBV collection is optional, as defined in Section 4.2.2.
[i] Pulmonary function tests must have occurred within 1 year of CRT Start. Results of testing performed at Wake Forest sites or other outside facilities is acceptable.

8.0 Specimen Collection and Storage

Detailed descriptions of sample collection, aliquoting and storage are described in Section 4.0. Exhaled breath condensate samples will be aliquoted, stored and secured within a locked freezer at -80° C until analysis.

Exhaled breath volatiles require immediate processing and analysis due to the volatile nature of the sample. As such, these will be batched and processed within 1-2 weeks after collection. Subsequent to this process, no sample will remain in existence. The data generated will be kept in a secure location according to Section 4.3.3 for future exploratory analyses.

Blood samples will be obtained in coordination with routine blood draws that occur as a part of standard of care for patients receiving CRT. Baseline blood will be obtained at the time of CT simulation requiring an additional phlebotomy procedure. The 2-week and 6-week blood samples will be obtained at the time of routine weekly labs (as part of standard monitoring during CRT). The 1-month blood will be obtained at the post-CRT visit, which occurs routinely as part of workup and confirmation of eligibility for consolidation immunotherapy.

To facilitate coordination between breath and blood collection, breath samples will be obtained on the same day as the blood samples, on the day of CT simulation (pre-CRT baseline), on the day of 2-week and 6-week routine on-treatment blood draws, and at the 1-month visit post-CRT.

All samples will be de-identified and stored securely at -80° C in the laboratory of Dr. Andrew Bishop. Samples (non-EBV) will be stored until analysis and remaining samples will be retained indefinitely for future study.

Blood samples will be stored indefinitely in the laboratory of Dr. Andrew Bishop until analyzed. These samples will be de-identified by the unique study ID code given at the time of collection.

9.0 Data Management

Informed consent document	EPIC
Protocol registration form	WISER/OnCore
Medical History	WISER/OnCore
Specimen Collection Form	WISER/OnCore
Antibiotic and Steroid Use (Medications Form)	WISER/OnCore
Tobacco Use Form	WISER/OnCore

Radiotherapy Form	WISER/OnCore
Pneumonitis Form	WISER/OnCore
Research Data	Secure research lab computers and servers

10.0 Confidentiality and Privacy

Confidentiality will be protected by collecting only information needed to assess study outcomes, minimizing to the fullest extent possible the collection of any information that could directly identify subjects, and maintaining all study information in a secure manner. To help ensure subject privacy and confidentiality, only a unique study identifier will appear on the data collection form. Any collected patient identifying information corresponding to the unique study identifier will be maintained on a linkage file, store separately from the data. The linkage file will be kept secure, with access limited to designated study personnel. Following data collection, subject identifying information will be destroyed. Samples will be de-identified and stored indefinitely in a secured wake forest laboratory. Consistent with data validation and study design, producing an anonymous analytical data set. Data access will be limited to study staff. Data and records will be kept locked and secured, with any computer data password protected. No reference to any individual participant will appear in reports, presentations, or publications that may arise from the study.

11.0 Data Safety and Monitoring

The principal investigator will be responsible for the overall monitoring of the data and safety of study participants. The principal investigator will be assisted by other members of the study staff.

12.0 Reporting of Unanticipated Problems, Adverse Events or Deviations

Due to the observational nature of this study, adverse events of interest are limited to those that occur in the immediate duration of the non-invasive exhaled breath collection itself. Should an AE related to breath collection occur, the investigators will notify study staff for logging in the Adverse Event Log (Appendix L).

Any unanticipated problems, deviations or protocol changes will be promptly reported by the principal investigator or designated member of the research team to the IRB.

12.1 Adverse Event Definitions

12.1.1 Adverse Event (AE)

An adverse event (AE) is defined as any unexpected, unfavorable or unintended condition that occurs immediately during the exhaled breath condensate or exhaled breath volatile collection (while the collection device is applied to the patient).

12.1.2 Serious Adverse Event (SAE)

A serious adverse event (SAE) is any untoward medical occurrence that occurs during the exhaled breath condensate or exhaled breath volatile collection (while the collection device is applied to the patient) that satisfies any of these criteria: results in death; is life-threatening; requires inpatient hospitalization or prolongs existing hospitalization; results in persistent or significant disability or incapacity or substantial disruption of the ability to conduct normal life functions; or if the event results in a congenital anomaly or birth defect.

12.2 Recording Adverse Events

When an AE occurs, it is the responsibility of the Investigator to review all documentation (e.g., medical progress notes, laboratory, and diagnostics reports) relative to the event. The study staff will then record all relevant information regarding an AE in the AE Log (Appendix L).

12.3 Adverse Event Characteristics

- **CTCAE term (AE description) and grade:** The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 5.0. A copy of the CTCAE version 5.0 can be downloaded from the CTEP web site (http://ctep.cancer.gov).

- **'Expectedness'**: AEs can be 'Unexpected' or 'Expected' (see Section 7.1 above) for expedited reporting purposes only.

- **Attribution** of the AE:

- Definite – The AE **is clearly related** to the study collection.
- Probable – The AE **is likely related** to the study collection.
- Possible – The AE **may be related** to the study collection.
- Unlikely – The AE **is doubtfully related** to the study collection.
- Unrelated – The AE **is clearly NOT related** to the study collection.

12.4 STRC SAE Reporting Requirements

The Data Safety Monitoring Committee (DSMC) is responsible for reviewing SAEs for WFBCCC Institutional studies as outlined in Appendix N. All Adverse Events that occur during protocol intervention and are coded as either 1) unexpected grade 4, 2) unplanned inpatient hospitalization ≥ 24 hours (regardless of grade), or grade 5 (death) must be reported to the DSMC using the using the SAE console in WISER.

All WFBCCC Clinical Protocol and Data Management (CPDM) staff members assisting a Principal Investigator in investigating, documenting and reporting an SAE qualifying for DSMC reporting are responsible for informing a clinical member of the DSMC as well as the entire committee via the email notification procedure of the occurrence of an SAE.

12.5 WFUHS IRB AE Reporting Requirements

Any unanticipated problems involving risks to subjects or others and adverse events shall be promptly reported to the IRB, according to institutional policy. Reporting to the IRB is required regardless of the funding source, study sponsor, or whether the event involves an investigational or marketed drug, biologic or device. Reportable events are not limited to physical injury, but include psychological, economic and social harm. Reportable events may arise as a result of drugs, biological agents, devices, procedures or other interventions, or as a result of questionnaires, surveys, observations or other interactions with research subjects.

All members of the research team are responsible for the appropriate reporting to the IRB and other applicable parties of unanticipated problems involving risk to subjects or others. The Principal Investigator, however, is ultimately responsible for ensuring the prompt reporting of unanticipated problems involving risk to subjects or others to the IRB. The Principal Investigator is also responsible for ensuring that all reported unanticipated risks to subjects and others which they receive are reviewed to determine whether the report represents a change in the risks and/or benefits to study participants, and whether any changes in the informed consent, protocol or other study-related documents are required.

Any unanticipated problems involving risks to subjects or others occurring at a site where the study has been approved by the WFUHS IRB (internal events) must be reported to the WFUHS IRB within 7 calendar days of the investigator or other members of the study team becoming aware of the event.

Any unanticipated problems involving risks to subjects or others occurring at another site conducting the same study that has been approved by the WFUHS IRB (external events) must be reported to the WFUHS IRB within 7 calendar days of the investigator or other members of the study team becoming aware of the event.

Any event, incident, experience, or outcome that alters the risk versus potential benefit of the research and as a result warrants a substantive change in the research protocol or informed consent process/document in order to insure the safety, rights or welfare of research subjects.

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Appendix A – Subject Eligibility Checklist

IRB Protocol No. IRB00059924	**WFBCCC Protocol No. 98119**
Study Title: A Pilot Study of Exhaled Breath Analysis to Predict Risk of Symptomatic Pneumonitis After Chemoradiotherapy For Stage III Non-Small Cell Lung Cancer	
Principal Investigator: Michael Farris, MD	

Inclusion Criteria (as outlined in study protocol)	**Criteria is met**	**Criteria is NOT met**	**Source Used to Confirm * (Please document dates and lab results)**
Age greater than or equal to 18 years old	☐	☐	
Clinically diagnosed or suspected Stage III non-small cell lung cancer to be treated with chemo-radiotherapy as part of cancer treatment, as determined by the treating clinician	☐	☐	
Plan for treatment with definitive radiotherapy (≥60 Gy) with concurrent chemotherapy at the discretion of the treating radiation and medical oncologists.	☐	☐	
Willing and able to tolerate exhaled breath collection	☐	☐	
Able to provide informed consent	☐	☐	
Exclusion Criteria (as outlined in study protocol)	**Criteria NOT present**	**Criteria is present**	**Source Used to Confirm * (Please document dates and lab results)**
Systemic (oral, intravenous or intramuscular) corticosteroid use for any reason within 5 days of registration.	☐	☐	
Prior radiotherapy directed at the chest (thoracic inlet superiorly to diaphragm inferiorly).	☐	☐	
Any antibiotic use within 2 weeks of registration	☐	☐	

This subject is ☐ eligible / ☐ ineligible for participation in this study.

OnCore Assigned PID: ___ ___ ___ ___ ___ ___ ___ ___

Signature of research professional confirming eligibility: _____

Date: ___ ___/___ ___/___ ___

Signature of Treating Physician**: _____

Date: ___ ___/___ ___/___ ___

* Examples of source documents include clinic note, pathology report, laboratory results, etc. When listing the source, specifically state which document in the medical record was used to assess eligibility. Also include the date on the document. Example: “Pathology report, 01/01/14” or “Clinic note, 01/01/14”

**Principal Investigator signature can be obtained following registration if needed

Appendix B – Protocol Registration Form

DEMOGRAPHICS

Patient: Last Name: _____ First Name: _____

MRN: ___ ___ ___ ___ ___ ___ ___ ___ DOB (mm/dd/yy): ___ ___ / ___ ___ / ___ ___

ZIPCODE: ___ ___ ___ ___ ___

SEX: ☐ Male ☐ Female Ethnicity (choose one): ☐ Hispanic ☐Non-Hispanic

Race (choose all that apply): ☐ WHITE ☐BLACK ☐ ASIAN ☐ PACIFIC ISLANDER ☐ NATIVE AMERICAN

Height: ___ ___.___ inches Weight: ___ ___ ___.___lbs.(actual)

Surface Area: ___ ___.___m^2

Primary Diagnosis: _____

Date of Diagnosis: ___ ___ /___ ___/___ ___

Performance Status: ___ ___ ___ ☐ ECOG ☐ Karnofsky

PROTOCOL INFORMATION

Date of Registration: ___ ___ / ___ ___ / ___ ___

MD Name (last) : _____

Informed written consent: (consent must be signed prior to registration) ☐ YES ☐ NO

Date Consent Signed: ___ ___ / ___ ___ / ___ ___

PID # (to be assigned by OnCore): ___ ___ ___ ___ ___ ___ ___ ___

Protocol Registrar can be contact by calling 336-713-6767 between 8:30 AM and 4:00 PM, Monday – Friday.

Completed Eligibility Checklist and Protocol Registration Form must be hand delivered, faxed or e-mailed to the registrar at 336-7136772 or registra@wakehealth.edu.