Investigation of Microbiomes of Postmenopausal Women Looking for Outcomes and Response to Estrogen Therapy

Study Protocol & Statistical Analysis Plan

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IMPLORE Study: **I**nvestigation of **M**icrobiomes of **P**ostmenopausal Women **L**ooking for **O**utcomes and **R**esponse to **E**strogen Therapy

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A. PROPOSAL OVERVIEW

The significant impact of vulvovaginal atrophy (VVA) is well recognized as approximately half of women will experience this disorder at some point in the postmenopausal years (1, 2). Symptoms like vaginal dryness, itching, burning, dyspareunia, vaginal bleeding or a combination of these are often encountered. This translates to "sizeable proportions of women who report emotional, lifestyle, and sexual impact from these symptoms (3)." The number of women experiencing this disorder will continue to grow as the older proportion of the population is expected to double in the next 30 years (4). However, the full extent of this impact is difficult to estimate as this disorder is multifactorial and often underreported (5). VVA, as part of a larger diagnosis of Genitourinary Syndrome of Menopause (GSM), is associated with concomitant genitourinary conditions and women with VVA have a higher incidence of overactive bladder and lower urinary tract symptoms (6, 7). Thus improvement in VVA can have a very real consequence on the functional status and quality of life in affected women.

In this proposed pilot study, 16S ribosomal RNA (rRNA) gene sequencing will be used in the analysis of microbiomes in postmenopausal women before and after eight weeks of vaginal estrogen use. We plan to characterize the composition and dynamics of the microbiomes of the vagina, bladder, and rectum for quantitative and qualitative changes in the distribution of operational taxonomic units (OTUs) before and after eight weeks of local vaginal estrogen therapy. Although the vagina, bladder, and gut microbiomes have been increasingly independently studied, less is known about the interactions of the bacterial communities among the three environments as well as the dynamic relationship with menopausal status and transvaginal estrogen therapy.

AIM 1: To assess the quantitative change in relative abundance of Lactobacillus genus and spp among the community composition of bacteria in the vagina pre- and post- vaginal estrogen therapy.

AIM 2: To evaluate the vaginal maturation index (VMI), vaginal pH, and vaginal inflammatory biomarkers: IL-1B, IL-4, IL-6, IL-8, IL-10, TNF-α, MCP-1, GM-CSF and correlate any notable changes with changes noted in the vaginal microbiome.

AIM 3 and 4: To observe for quantitative and qualitative changes in the distribution of OTUs among the bladder microbiome (AIM 3) and the rectal microbiome (AIM 4).

Sub Aims 3 and 4: To assess for quantitative changes in bladder and rectal inflammatory biomarkers and correlate these findings both with any notable changes in the bladder and rectal microbiomes as well as with the data yielded from vaginal sampling.

Based on limited prior research from Shen J, et al. we hypothesize that transvaginal estrogen therapy will result in significant increases in Lactobacillus species with corresponding decreases in gram negative and anaerobic species. Therefore, vaginal estrogen therapy will also yield a decrease in overall bacterial diversity with an expected Lactobacillus predominance. Our hypothesis is currently unstudied and to our knowledge there has not been a study of 16S rRNA gene sequencing of the vaginal microbiome with local vaginal estrogen therapy in any manner.

This proposed research is critical to evaluating the relationship between VVA and these dynamic site-specific microbiomes. Furthermore, this proposal allows us to evaluate the microbiome response to vaginal estrogen therapy – a widely used treatment approach to patients with VVA. This information will also inform future studies, and serve to guide estimations of power and necessary cohort numbers. Currently the baseline and post treatment compositions of these microbiomes are unclear in our study population of interest and this pilot study will help to define that. If our hypotheses are confirmed, future studies from samples collected in this pilot study will allow us to investigate the relationships of rectal, urinary, and vaginal metabolites in VVA and its associated physiologic and inflammatory changes. Furthermore, alternative prophylactic uses of vaginal estrogen for prevention of lower genitourinary tract conditions may also be explored.

This study will utilize multidisciplinary expertise from the University of Alabama at Birmingham among the departments of Urogynecology, UAB Microbiome Resource, Pathology, Maternal Fetal Medicine, the Center for Clinical and Translational Science, and the Center for Women's Reproductive Health.

B. SIGNIFICANCE

B.1. Significance of Vulvovaginal Atrophy: The significant impact of vulvovaginal atrophy (VVA) is well recognized as approximately half of women will experience this disorder at some point in the postmenopausal years (1, 2). Symptoms like vaginal dryness, itching, burning, dyspareunia, vaginal bleeding or a combination of these are often encountered. This translates to "sizeable proportions of women who report emotional, lifestyle, and sexual impact from these symptoms (3)." The number of women experiencing this disorder will continue to grow as the elderly proportion of the population is expected to double in the next 30 years (4).

However, the full extent of this impact is difficult to estimate as this disorder is multifactorial and often underreported (5).

B.2. Relationship of VVA to Other Genitourinary Conditions: VVA, as part of a larger diagnosis of Genitourinary Syndrome of Menopause (GSM), is highly associated with concomitant genitourinary conditions and women with VVA have a higher incidence of overactive bladder and lower urinary tract symptoms (6, 7). Some women have been shown to have increased episodes of urgency urinary incontinence (UUI) despite no clinical or culture evidence of bacterial UTI but with positive urinary bacteria detected by quantitative PCR (8).

B.3. Pathophysiology of VVA and Hypoestrogenemic State: The pathophysiology of the relationship between a hypoestrogenemic state and changes in the vulvovaginal area have been described. Significant changes in vaginal pH, cellular glycogen stores and integrity, adipose tissue and vaginal secretions have been observed (9-11). Decreased levels of estrogen have also been shown to impact the local cellular immunity of the vagina leading to increased levels of neutrophils, cellular destruction and inflammation (9).

B.4. Relevance of Interest in Microbiome Niches: Symbiotic and dysbiotic relationships have been described as they pertain to protective and pathogenic effects on the host. Vaginal microbiomes of postmenopausal women have been shown to have increased rates of anaerobic, mixed flora communities and decreased predominance of lactobacillus species (13). This has been associated with increased alkaline pH values of the vagina that make for a less suitable environment to the lactobacillus spp. Interest in human microbiomes has existed for more than a century. From microscopy to Gram stains and cultures, the analysis of microbiomes has evolved to culture independent next-generation gene sequencing (14). These molecular techniques for analysis of human microbiomes have developed over decades and 16S rRNA gene sequencing for analysis has been shown to be a valid tool for analyzing distributions of OTUs (14-16).

B.5. Effect of Microbiome Niches on Host Well-being: In addition to the microbiomes of the vagina and bladder in post-menopause, which have been increasingly studied, there is great interest in the relationship of the microbiomes of the gastrointestinal tract and rectum and how it relates to the genitourinary environments. A study concluded that Lactobacilli colonizing the rectum may be a reservoir for vaginal lactobacilli (17). A study found significant associations between menopause, hypoestrogenemia, disorders of the GI tract and oral cavity as well as alterations of both the oral and gut microbiomes that lead to dysbyosis and loss of health integrity (18). Inflammatory states of the gut and autoimmune conditions have been associated with gut microbial dysbyosis. Bone homeostasis has also been implicated in the sphere of influence of gut microbiota (18).

B.6. Standard Treatment of VVA: In women without contraindications, vaginal estrogen therapy, among other treatment options, is considered a standard of care for treatment of vulvovaginal disorders including vaginal atrophy, dyspareunia, and some lower urinary tract symptoms (19-21). Vaginal estrogen therapy has been shown to yield a statistically significant increase in the VMI and decreases in both the vaginal pH and severity of the reported most bothersome symptom (MBS) (23). This study, however, did not concurrently evaluate any effect of estrogen therapy on vaginal bacterial communities and questions still remain regarding how vaginal estrogen therapy specifically affects the composition of the microbiome and how these compositional changes affect physiologic change.

B.7. Limitations of Current Knowledge: Our current knowledge is based on assumptions from other limited studies as well as extrapolated from research evaluating the vagina both in pre- and post-menopausal states or with other methods of estrogen delivery (24). The prevailing theory is that with decreasing estrogen levels in the aging woman, we see decreasing levels of glycogen stores in the vagina (both intra and extracellular) which leads to decreases in Lactobacillus species, decreased H_20_2 presence in the vagina, increases in vaginal pH, and increased biodiversity including various gram negative, coliform and anaerobic species (25). While aspects of these theories are documented in the literature other associations have been noted as well, some reporting different results. A recent study assessing correlations between levels of vaginal glycogen did not support an association with quantities of lactobacillus dominant microbiomes (26). This same study also did not note an association between documented Lactobacillus predominant microbiomes and fewer VVA symptoms. With this in mind, the findings from our study will be pertinent to further interpreting these varied pathophysiologic associations.

C. INNOVATION

16S rRNA gene sequencing is a well validated and increasingly predominant mode of evaluating bacterial communities among locations of interest (14-16). To our knowledge, there has not been a study of the vaginal microbiome and any assessment of its relationship to therapy with intravaginal estrogen cream utilized prospectively. Further, there are limited studies that evaluate changes in the vaginal microbiome in a

pre- and post- manner after any of the other routes estrogen therapy. The same holds true for evaluation of the bladder and gut microbiomes in response to vaginal estrogen therapy. Additionally, there is limited data combining efforts to assess the dynamic relationships of these microbiomes with local inflammatory biomarkers and standard clinical tools to evaluate vaginal health.

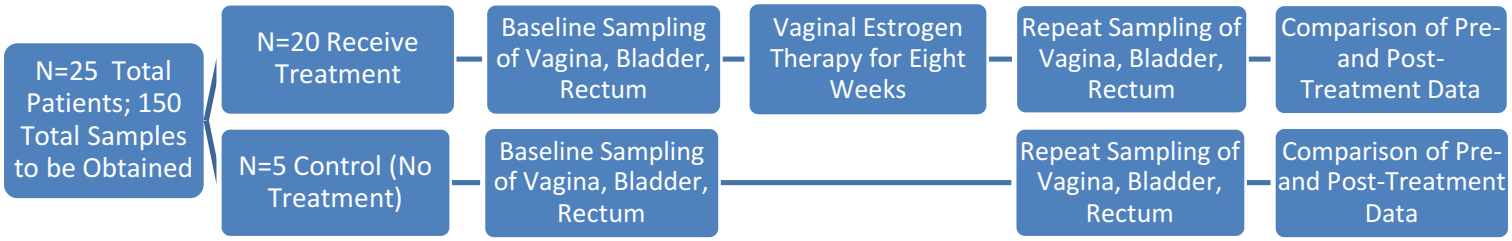
Assessing the interrelationships of these microbiomes and local inflammatory biomarkers is crucial to understanding how pathologic or therapeutic changes in these markers affect the other microbiome niches. Given that the microbiomes of these three environments are conceivably modifiable, the understanding of the correlative relationship between the microbiomes and inflammatory biomarkers as well as objective improvement of the vagina can provide important clarity and guidance for future research and potential therapies. Results from this study will also serve to guide estimations of power and necessary cohort numbers. Currently the baseline and post treatment compositions of these microbiomes are unclear in our study population of interest and this pilot study will help to define that. If our hypotheses are confirmed, future studies from samples collected in this pilot study will allow us to investigate the relationships of rectal, urinary, and vaginal metabolites in VVA and its associated physiologic and inflammatory changes. Furthermore, alternative prophylactic uses of vaginal estrogen for prevention of lower genitourinary tract conditions may also be explored.

D. METHODS

D.1 Study Population

D.1.1. Cohort Identification: Postmenopausal women ≥55 years old with clinically diagnosed vulvovaginal atrophy. Twenty volunteer patients will be placed in the intervention arm. The primary aim is to compare the change in the data points of interest over the eight week study period in a pre- and post-treatment comparison manner. The five patients meeting the same inclusion/exclusion criteria will serve as a control cohort, not to receive treatment, and are included in this study to demonstrate microbiome stability across the eight week time period. We anticipate interest and participation in this study from planned advertisements and women seen in the UAB Urogynecology and Women's Reproductive Health Clinics.

Figure 1. Flow Diagram of Enrollment and Study Design



Screening/Eligibility: During screening, eligibility will be determined using scripted questions: Inclusion/Exclusion Criteria. Potential subjects will be invited to participate in the study, demographics will be recorded. **Inclusion Criteria:** Postmenopausal women with clinically diagnosed vulvovaginal atrophy, age ≥55 years old and a screening vaginal pH of ≥5, without menses for ≥12 months, no uterovaginal or vaginal vault prolapse beyond the hymen. **Exclusionary Criteria:** 1) Patients with BMI ≥35kg/m^2. 2) Any patients with infections requiring antibiotic or antifungal therapy during the study period or within the last month or during the study period. 3) Study patients may not use any vaginal suppositories, douches, or vaginal hygiene wipes within the month preceding enrollment. 4) Patients receiving ongoing hormone replacement therapy. Patients already on hormone therapy, will be allowed to undergo a "wash out" period of estrogen or progesterone products for one month prior to enrollment. 5) Patients requiring concurrent use of steroid creams for other indications (e.g. lichen sclerosis). 6) Additional exclusions included patients with systemic conditions requiring immunosuppressive drugs, currently receiving chemotherapy, or history of pelvic radiation. 7) Any patients with contraindications to vaginal estrogen therapy including vaginal bleeding of unknown etiology. 8) Any patients with history of connective tissue disease. 9) Current tobacco use.

D.2. Study Design

The intention of this study is for each of the subjects to serve as her own control. Twenty patients will receive the treatment intervention. Five will not receive the treatment intervention. The five subjects not receiving

treatment are intended to serve as a control cohort to demonstrate stability of the microbiome over the study period. It is anticipated that unlike premenopausal subjects, the postmenopausal subjects not undergoing treatment (control group) will have little variation of the microbiome over the eight weeks. Each of the twenty-five subjects will be sampled for microbiome and inflammatory biomarker analysis at three different locations (vagina, bladder, rectum) at two separate time points (Baseline Visit 1 and after eight weeks of vaginal estrogen therapy, Visit 2). This will yield a total of 300 samples for the study. The treated subjects (20) and untreated (5) subjects may be compared to each other but that is not the primary aim of this study.

Table 1. Description of Sample Numbers and Locations.

	Pre-Treatment	Post-Treatment
SAMPLE LOCATION		
VAGINA		
Inflammatory Biomarkers	25	25
Microbiome	25	25
BLADDER		
Inflammatory Biomarkers	25	25
Microbiome	25	25
RECTUM		
Inflammatory Biomarkers	25	25
Microbiome	25	25

Intervention: 1g conjugated estrogen (CE) cream given nightly for 2 weeks then twice-weekly to complete planned eight weeks therapy. This specific dose and frequency has been shown to improve objective and subjective measures of vaginal epithelium in previous studies (23, 27). It has also been shown to achieve objective improvements in as little as three to four weeks (23). Additionally, this dose and regimen is already commonly used and will provide excellent external generalizability.

D.3. Specific Aims

D.3.1. Specific Aim – 1: To assess the quantitative change in relative abundance of Lactobacillus among the community composition of bacteria in the vagina pre- and post- vaginal estrogen therapy. ***Hypothesis:*** A statistically significant quantitative increase in the relative abundance of Lactobacillus will be seen.

Approach: The vaginal microbiome will be analyzed using 16S rRNA gene sequencing techniques evaluating specimens of mid-vaginal secretions to compare samples from each subject prior to initiation of vaginal estrogen cream and then again after eight weeks of therapy. This will yield 50 vaginal specimens in total for this specific aim (V1 = 25, V2 = 25; Treatment Group V1 = 20 vs. V2 = 20; Control Group V1 = 5 vs. V2 = 5). Baseline characteristics will be recorded and compared among the total cohort to identify covariates for adjusted analyses. Whole genome DNA (gDNA) will be extracted from specimens using DNA extraction kits (Zymo Research Corp, Irvine, CA) and PCR primers targeting the V4 rRNA gene will be used to prepare an amplicon library sequenced by the MiSeq platform (Illumina: San Diego, CA) and analyzed using the Quantitative Insight into Microbial Ecology (QIIME) suite. These raw data sequences will then be cross-validated and additional quality control measure will be applied. Analysis using QIIME will be supplemented with the use of the program DADA2 for more robust methods of sequence read de-noising and clustering to yield a higher degree of species-level identification (28). To better detect species of organisms known to be present in vaginal samples, taxonomy will be assigned using appropriately modified and validated databases (29-31) ***Microbiome Analysis Statement of Rigor:*** Several quality controls are performed: 1) removing sequences with low quality scores, those matching human databases and low-duplicates and 2) merging paired reads and discarding sequences with substantial differences. Results from the UAB Microbiome Resource pipeline showed that duplicate samples had significant co-clustering on Principal Coordinate Analysis (PCoA) plots. Using 16S rRNA gene and whole genome DNA sequences from 20 known species, we are able to correctly identify organisms with unique 16S rRNA V4 genes to the genus and even species level and predict genetic pathways. ***Sample Size and Power Rationale:*** Our sample size calculation is based upon our primary aim to evaluate the expected quantitative change in relative abundance of Lactobacillus genus and spp among the community composition of bacteria in the vagina. There's an abundance of research reporting the normalization of the vagina by objective measures by various routes of estrogen therapy, specifically by serving to facilitate the increase of the relative Lactobacillus presence. However, it is difficult to define how much of an increase in Lactobacillus is needed to be clinically relevant. As previously stated there is no previous study by which to reference in predicting what the expected change in Lactobacillus may be in the setting of vaginal estrogen therapy. A previous study (22) has compared a cohort of atrophic vaginitis (AV) patients who received low dose oral estrogen therapy to a healthy control (not receiving therapy); all patients were postmenopausal meeting strict criteria. Vaginal sampling took place at baseline, two weeks,

and at four weeks after treatment initiation. Among the AV group the relative abundance of Lactobacillus pretreatment was 0.112 ±0.164. At 4 weeks of oral estrogen therapy the Lactobacillus abundance rose dramatically to 0.71 ±0.375. The baseline abundance among the "healthy" untreated postmenopausal cohort was 0.532 ±0.403 and virtually without change over the four week period.

Based on these results, we expect the relative abundance of Lactobacillus in our cohort to be somewhere between 0.112-0.532 at baseline. As the selection criteria of our cohort does not include VVA symptomatology, we expect the relative abundance of Lactobacillus spp to be approximately 0.4 ±0.3.**With a Type-1 error of 0.05, we estimate with 80% power that we can detect an effect size of 0.2 increase (0.4→0.6) in the relative abundance of Lactobacillus spp with a treatment sample size of 20 patients.**

Biostatistical Analysis Plan: The following microbial community-level analyses will be performed to investigate the overall microbiome differences between the pre-treatment and post-treatments groups using the R package **phyloseq** (Table 2)(32).

Table 2. Overview of Community-Level Microbiome Analysis

Outcome	Measurements	Two-group comparisons
Alpha diversity (diversity within a sample)	Shannon, Chao, Simpson indices	t-tests, Mann-Whitney U
Beta diversity (diversity between samples, overall microbiota composition)	Bray-Curtis, unweighted UniFrac, weighted UniFrac (represented by PCoA to visualize clustering)	PERMANOVA or Dirichlet-multinomial distribution with Wald-type test

To identify individual taxa significantly associated with vaginal estrogen treatment, taxa-specific analyses will be performed. Given the distinct properties of microbiome data (compositional counts/proportions, varying total sequence reads across samples, over-dispersion), microbiome counts, transformations of microbiome counts, and beta regression for relative abundance will be used (33-39) To deal with zero-inflation of microbiome data, we will employ zero-inflated models: negative binomial (for count data), Gaussian (for logarithm or arcsine square root transformations), beta regression (for relative abundance) – which allow analysis of multiple covariates which may vary between treatment and non-treatment groups (38, 40-43). An R package *bmzim* for these models will be used. Standard procedures (e.g. Holm's, Hochberg's, Hommel's, Benjamini and Hochberg's methods) will be used to calculate p-values adjusted for multiple comparisons.

D.3.2. Specific Aim – 2: To evaluate the VMI, vaginal pH, and vaginal inflammatory biomarkers: IL-1B, IL-4, IL-6, IL-8, IL-10, TNF-α, MCP-1, GM-CSF and correlate any notable changes with changes noted in the vaginal microbiome. ***Hypothesis:*** An overall measured decrease in the levels of vaginal inflammatory biomarkers in response to therapy as well as an increase of the VMI with concomitant lowering of the vaginal pH, all signifying a return of the vaginal environment to a healthier state will occur. These changes will be highly correlated with increased Lactobacillus predominance.

Approach: The VMI, vaginal pH, and vaginal inflammatory biomarkers: IL-1B, IL-4, IL-6, IL-8, IL-10, TNF-α, MCP-1, GM-CSF will be collected at the same time points (V1 and V2). Changes in these data points will be correlated with changes noted in the vaginal microbiome (see below). As was used in a previous study (27), the VMI served as a tool for measure of adherence to vaginal estrogen application but also as an objective standard by which to correlate changes in the vaginal microbiome.

Biostatistical Analysis Plan: Mean inflammatory marker concentrations and VMI scores will be compared by Student t-test or robust non-parametric tests between pre- and post-treatment samples. Normal linear regression will also be used to test the difference of the two cohorts (cytokine concentrations as response, cohort indicator as covariate). Similar methods will be used to analyze the difference in cytokine levels from V1 and V2 between groups. We will use three methods to investigate the relationship between bacterial taxa and cytokine levels: 1) Pearson's correlation coefficients will be calculated between cytokine levels and bacteria taxa (measured by percentage of reads) with a heat map showing correlations that are significantly different between pre- and post-treatment groups. To determine if cytokine levels and microbiota contribute to the clustering of women pre- and post-treatment, PCoA will be used to determine if these groups separate in space. Clusters will be examined for potential novel subsets of samples - with clinical data used to identify the cause of the clustering with linear and discrete data methods. 2) We will treat counts of each taxa as response, inflammatory cytokine levels as covariates and use negative binomial models (or zero-inflated versions). 3) We will treat cytokine levels as response, relative abundance of each taxa as covariates and use linear models. In these regressions, a pre-treatment indicator and an interaction term between pre-treatment and the covariates will be included. Similar methods will be used to analyze VMI and vaginal pH. ***Sample Size and Power Rationale:*** Cytokine levels are continuous variables and even given this small sample size, should allow for more than adequate power to detect large differences in mean concentrations between pre- and post-treatment groups.

D.3.3. Specific Aims – 3 and 4: To observe for quantitative and qualitative changes in the distribution of OTUs among the bladder and rectal microbiomes. ***Hypothesis:*** Alterations in the bladder and rectal

microbiomes will occur before and after transvaginal therapy (similar to the changes seen in the vaginal microbiome). These specific aims are exploratory.

D.3.3.1 Sub Aims – 3.1 and 4.1 (Exploratory): To assess for quantitative changes in the bladder and rectal inflammatory biomarkers (IL-1B, IL-4, IL-6, IL-8, IL-10, TNF-α, MCP-1, GM-CSF) and correlate these findings both with any notable changes in the bladder and rectal microbiomes. Furthermore, changes in these biomarkers will be compared with the data (inflammatory and microbial) yielded from vaginal sampling. ***Hypothesis:*** We hypothesize that an overall measured decrease in the levels of bladder and rectal inflammatory biomarkers in response to therapy will occur.

Approach: The bladder and rectal microbiome samples will be obtained and analyzed at the same time points as Specific Aim 1. Bladder sampling will be via straight catheterization and rectal sampling will be via rectal swabbing (swab placed high into the rectum). Urine dipstick point of care testing will be performed for each baseline assessment and results suspicious for acute infection will be excluded from the study. Inflammatory biomarker testing will be performed and analyzed in the same manner as Specific Aim 2 from the bladder and rectal samples at the same time points and changes will be statistically correlated with bladder and rectal sampling, as well as the vaginal sampling. The biostatistical analysis plan will follow the plan outlined independently for Aims 1 and 2.

D.4 Potential Problems/Alternative Strategies and Benchmarks for Success: The main problem we may encounter during this investigation is that not all taxonomic differences can be resolved to the genus or species level. Therefore, it is possible to find statistically significant differences in certain taxa for which there is no identification in the taxonomic databases. However, these data can be continuously reanalyzed with expanding taxonomic databases in the future to add to our findings.

Our benchmarks for this study are presented in the timeline shown below. These benchmarks are dependent on the streamlined process for microbiome analysis that has been established. DNA extraction will be done by Dr. Elena Lobashevsky, who has previously extracted DNA for our department's microbiome studies; DNA amplification and sequencing will be done by Dr. Morrow and colleagues; statistical analysis will be performed by the UAB Microbiome Bioinformatics and Biostatics division (led by Dr. Elliot Lefkowitz with Dr. Nengjun Yi as the lead biostatistician.) This collaboration allows all sequencing and analysis to be performed at one site in a timely fashion.

E. TIMELINE: As noted in the separate document describing the institutional and departmental environment, the University of Alabama at Birmingham has a long track record of successfully completing clinical and translational trials. As such, the proposed study is feasible within the predicted time frame. The estimated time frame to accomplish these aims is illustrated below.

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