Title: Endocrine, Metabolic and Microbiome Influence on the Post-COVID Syndrome

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Background and Significance

The onset of the COVID-19 pandemic has led to a subset of patients that, once recovered from the acute infection, also experience an intractable and debilitating set of lingering symptoms termed post-COVID syndrome, also known as post-acute sequelae of SARS CoV-2 (PASC). The most common symptoms include anxiety, shortness of breath, continued loss of the sense of smell and taste, loss of appetite with subsequent weight loss, sleep difficulties, severe fatigue, cognitive dysfunction (foggy brain) and increased frailty. These patients frequently present to the emergency room looking for symptom management because they are unable to perform normal activities of daily living and maintain job performance. The mechanisms behind this syndrome have proven elusive because patients symptom severity and treatment regimen including different requirements for hospitalization, supplemental oxygen, dexamethasone and remdesivir. Thus, it is critical to characterize the baseline endocrine, metabolic, inflammatory and microbiome alterations in PASC patients to better identify and manage the symptoms to prevent potential long-term health consequences.

UTMB has established a post-COVID clinic for management of PASC patients, but it is recognized that a more complete clinical picture of the underlying mechanisms driving these lingering symptoms is needed. UTMB's post-COVID clinic has seen over 150 patients to date with a waiting list of over 200 patients who wish to be treated. Thus, it is clear that there is great demand for understanding the mechanisms and potential treatments for the long-term debilitating effects of PASC.

Persistent and long-lasting health problems are common in patients after COVID-19 infection. In a recent study of patients that had been hospitalized with COVID-19, two months after discharge, 87% reported at least one lingering symptom (joint pain, fatigue, breathing issues, etc), more than 50% reported

more than three lingering issues, and over 40% reported a reduction in their of quality of life. Another study found that at 1-month after hospitalization for COVID-19, 74% reported persistent issues related to shortness of breath and a decrease in both physical and mental health. Preliminary data from our Post-COVID Recovery clinic agree with these two recent reports. In our study, 1 1/2 months after COVID-19 diagnosis, patients reported on average 10 of the 18 common symptoms (with 90% having chest pain, 87% dyspnea, 75% fatigue, and 90% with cognitive changes). While the previous studies examined patients that had severe COVID-19 infections, >50% of our patients were never hospitalized, yet have numerous persistent symptoms. This has serious implications for the ability of patients to return to work, downstream effects on mental health due to sometimes drastic lifestyle and work capacity changes, and the ability to engage in activities or hobbies enjoyed prior to COVID-19 illness.

Notably, the cluster of symptoms associated with PASC include profound fatigue and cognitive dysfunction, which are strikingly consistent with a syndrome that our clinical research team has described in patients after traumatic brain injury (TBI) designated BIAFAC (Brain Injury Associated Fatigue and Altered Cognition). Over the last 12 months we have reported the characteristics of BIAFAC syndrome (Urban 2020; Yuen et al. 2020). In particular, TBI patients with BIAFAC present with lingering and profoundly debilitating symptoms including severe fatigue, cognitive dysfunction (foggy brain), sleep disturbances, and the inability to perform activities of daily living that persist for years post-injury. Mechanistically we have explored the role of the gut microbiome discovering altered communities in TBI patients in long-term care facilities compared with controls (Urban et al. 2020). We also established that many TBI patients with BIAFAC also present with abnormal growth hormone (GH) secretion, and when treated with recombinant GH, a majority of patients have significant improvement of both fatigue and impaired cognition (Wright et al. 2020). While studies are underway to understand the details of the mechanism causing BIAFAC and why GH treatment alleviates symptoms in these patients, we are intrigued that the symptom phenotype of PASC patients overlaps with many BIAFAC symptoms. It is possible that PASC symptoms may be addressed through similar treatment strategies including the potential for prebiotic/probiotic enhancement of microbiome health.

In the current pilot proposal, we will characterize the baseline endocrine, metabolic, inflammatory and gut microbiome alterations in PASC patients and compare those to our extensive database of BIAFAC patients and normal controls. From this critical baseline data, we will develop carefully defined clinical research trials that will test potential treatments for alleviating the syndrome. We **hypothesize** that an imbalanced endocrine axis stemming from COVID infection leads to metabolic, inflammatory and microbial dysregulation resulting in the onset of persistent PASC symptoms.

Specific Aims

Specific Aim 1: Characterize baseline measures of endocrine function, metabolism, inflammation, and composition of the gut and nasal microbiome of patients reporting PASC symptoms.

Specific Aim 2: Assess neuropsychological measures of fatigue, sleep, and cognition for patients reporting PASC symptoms.

Specific Aim 3: Compare physiological and neuropsychological measures of PASC patients to our extensive database of BIAFAC patients and normal controls.

Specific Aim 4: Characterize the microbiome of PASC patients and compare to: a)our database of healthy control subjects, b) our database of symptomatic BIAFAC patients, c) new collected samples of patients with a history of COVID who did not develop PASC.

Experimental Design and Methods

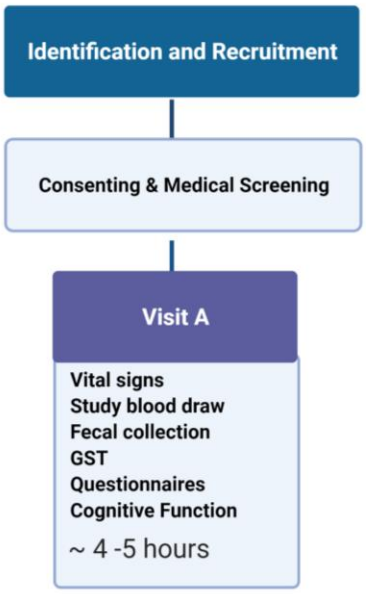
Subjects. We will study subjects (aged 18 - 80 years) with a history of COVID (n=16M/16F) that have been seen in the UTMB COVID clinic for ongoing symptom management and subjects (aged 18 – 80 years) with a history of COVID (n=16M/16F) who did not develop PASC. Potentially eligible patients may also self-refer to the study from surrounding post-COVID clinics or clinicaltrials.gov. Subjects with a history of COVID and no PASC will be recruited by placement of flyers around the community as well as through UTMB announcements.

(*See Protection of Human Subjects - Recruitment Methods and Consenting Process for detailed information regarding recruitment and consenting*).

COVID non-PASC Control Subjects

Interested subjects with a previous diagnosis of COVID without a history of PASC will undergo a phone pre-screen. If they are interested, they will be scheduled for a formal consenting and medical screening at the UTMB Clinical Research Center (CRC). Subjects will report to the UTMB Institute for Translational Sciences (ITS) Clinical Research Center (CRC) for the medical screening and one study visit. The study visit will consist of a glucagon stimulation test (GST) to assess growth hormone status; questionnaires assessing health status, mood and quality of life, diet, sleep, and gastrointestinal health. Blood samples will be drawn to measure amino acid levels, hormones, and metabolites. Fecal samples will be collected to analyze the GI microbiome.

Screening Procedures: All subjects consenting will undergo the following procedures. Screenings will take place at the UTMB ITS Clinical Research Center (ITS-CRC).



- Vital signs. Vital signs will be collected at screening. This will include height, weight, blood pressure, pulse, respirations, and temperature.

- Urine Screening Labs. Urine will be collected at screening and sent to the UTMB clinical laboratory for basic urinalysis. Results will be entered into the UTMB medical chart.

    - Urinalysis

- Urine Pregnancy Test. Urine will be collected from female subjects for a urine pregnancy test to be performed at the UTMB ITS-CRC as point of care testing (POCT). Women who are post-menopausal will be exempt from this test.

- COVID test. A PCR test using a nasal swab will be used to ensure that the subject is negative for active COVID virus before beginning study. Subjects with negative PCR COVID tests within 2 weeks of screening date will not be retested.

- Screening Blood Labs. For initial screening, a 20 ml of blood will be drawn using a butterfly needle from a peripheral vein in the arm. Blood will be sent to the UTMB clinical laboratory for analysis and results entered into the subjects UTMB medical chart. Laboratory results will include:

    - Complete blood count with differential (CBC w/ diff)
    - Comprehensive metabolic panel (CMP)
    - Lipid Panel
    - Hepatitis B surface antigen
    - Hepatitis C virus antibody
    - HIV screen

- History and Physical. Subjects will undergo a medical history and physical examination by a study nurse, medical student or physician. Recent (within 3 months) clinical history and physical may be used in place.

Study Procedures:

Visit A

- Vital signs. Vital signs will be collected. This will include height, weight, blood pressure, pulse, respirations, and temperature. Vitals will be collected both sitting and standing.

- Urine. A clean catch urine will be obtained. Urine will be frozen at -80C and used to measure proteinuria, urinary microbiome signatures and renal function.

- Phlebotomy. Subjects will have a catheter placed into a peripheral vein in their arm (0.9% sodium chloride set at TKO) for the glucagon stimulation test. The IV will be discontinued when the glucagon stimulation test is complete.
- Blood draw. Up to 20 ml of blood (2-serum separator top (5ml); 1-lithium heparin (6ml); 1-K-EDTA (4ml)) will be collected from fasted (at least 10 hours) subjects for analysis of hormones, amino acids and metabolites.

  An additional 20 ml of blood will be collected for the following labs to be sent to the UTMB clinical laboratory for analysis (all labs run at UTMB will be documented in the subject's UTMB medical chart) An additional 3.5 ml blood will be collected and frozen as a back up in case of a problem with labs sent to ARUP.:
  - Insulin-like Growth Factor 1 (IGF-1)
  - Follicle Stimulating Hormone (FSH)
  - Sex Hormone Binding Globulin (SHBG)
  - Total Testosterone
  - Free Testosterone
  - Prolactin
  - Thyroid Stimulating Hormone (TSH)
  - Free T4
  - High Sensitivity CRP
  - Vitamin B12
  - Vitamin D

- Glucagon stimulation test. Subjects will have a glucagon stimulation test. After the IV is placed, 3.5 ml of blood will be collected for the baseline (time: 0 minutes) to test for levels of human growth hormone. 1 mg glucagon (for subjects over 90 kg, 1.5 mg glucagon) will be injected IM in the deltoid muscle of the subject. Additional blood samples (3.5 mL) will be collected to test for levels of human growth hormone at time points: 90 minutes, 120 minutes, 150 minutes, and 180 minutes. All blood samples will be sent to UTMB clinical laboratory for analysis and entered into subjects UTMB medical chart.

- GI microbiome. The GI microbiome will be assessed using a self-collected fecal sample. Subjects will provide a fecal sample as described below. Samples will be stored at room temperature until transferred to UTMB (on/+ 5 days after collection). Subjects will be given a tutorial on how to properly collect the fecal sample. They will be given a handout from the manufacturer of the collection kit with step by step instructions. They will also be shown a video of the collection process supplied by the kit manufacturer.

  https://www.youtube.com/watch?v=ytr_hmJdHqM&feature=youtu.be&rel=0

  Fecal specimens will be collected using the OMNIgene-Gut® (OMR-200) collection kit from DNA genotek (please see product inserts attached as pdf files). To assist in collection, the additional toilet accessory will be employed where appropriate (OM-AC1). The manufacturer's instructions will guide sample collection. Briefly, fecal material will be collected by catch on a kit-provided tissue or toilet hat that has been wiped with a

disinfecting wipe prior to use. The study participant's fecal material will be transferred using the kit included spatula into the yellow top of a collection tube with the purple cap unscrewed. The tube will then be re-capped tightly and shaken for a minimum of 30 seconds to mix feces with kit included stabilization liquid. Storage recommendations as per manufacturer allow up to 60 days at room temperature or up to several months at -20°C or -80°C in the kit provided collection tube. Samples will be labeled with the subject's unique code and stored at room temperature until transported to University of Texas Medical Branch (UTMB). Samples will be processed and analyzed by qPCR as described below.

Nucleic acids will be extracted from batched fecal specimens using our optimized workflow that includes use of the DNeasy PowerMicrobiome kit (2600-50-1, Qiagen). If necessary, samples will be diluted/mixed 1:1 with sterile PBS to yield a pipettable liquid slurry. For each sample, 100 µl of the fecal liquid fraction will be mixed with 200 µl of kit-provided lysis buffer into a commercially available tube containing 0.1 mm glass beads (2600-50-BT, Qiagen). Samples will then be heated at 55°C for 30 minutes. Subsequently, samples will be homogenized for 5 minutes at 30 Hz using a Tissue Lyser instrument (Qiagen). Samples then will be centrifuged at 13,000 x g for 1 minute at room temperature. The liquid fraction will be placed into a sterile microtube along with 50 µl of IRS solution (2600-50-2, Qiagen) and incubated at 4°C for 5 minutes. The sample will then be centrifuged as before and the liquid fraction taken for extraction of DNA using a MagNAPure 96 automated nucleic acid extraction platform (Roche). Purified DNA will be used for next-generation sequencing and qPCR analyses to create molecular profiles for each microbiome. Residual DNA will be archived at -20°C.

An additional collection will be performed to collect viable fecal material. Fecal samples will be collected using the spatula included in the OMNIgene-Gut kit used in the first collection. Briefly, after the participant will transfer fecal material to the yellow top of the collection tube for the first collection, then they will scoop another small amount of fecal material with the same spatula and transfer it to a prepared cryogenic freezer tube containing sterile PBS (without Ca/Mg) and 10% sterile glycerol. The tube will be closed and shaken for a minimum of 30 seconds to mix with the stabilization liquid. Samples will be labeled with the subject's unique code and stored at 4°C until transported to University of Texas Medical Branch. Samples will then be frozen at -80°C and stored until the molecular analysis is complete. After initial analysis, if the sample is considered useful (representative of the average non-PASC control subject), it may be thawed, diluted in saline and used either to mix in a mathematical ratio to create a synthetic community or to be directly transplanted into germ free mice. If the sample is considered not useful (not representative of the average non-PASC control subject) for fecal microbiome transplant into germ free mice, the sample will be destroyed.

- Questionnaires. Questionnaires will be completed to assess perceptual fatigue, symptoms associated with growth hormone deficiency, depression, sleep, general quality of life, diet and gastrointestinal symptoms.

- **EuroQOL-5 Dimensions (EQ-5D)** is a 5 item assessment of health status measuring mobility, self-care, usual activity, discomfort or pain and depression or anxiety.

- **Medical Outcome Study - Short Form 36 (MOS-SF-36)** is a questionnaire developed by RAND Health to measure patient self-reported quality of life. Eight subscales are measured including physical function, role limitations due to physical health, role limitations due to emotional problems, energy/fatigue, emotional wellbeing, social functioning, pain and general health. Scores for each subscale range from 0 - 100, with 100 being better perceived health and 0 being worst perceived health.

- **Hospital Anxiety and Depression Scale (HADS)** is a 14 item assessment which includes 7 questions for anxiety and 7 questions for depression, which are summed separately for a total score for each. HADS classifies scores 0-7 as normal; 8-10 as borderline abnormal (borderline case); 11-21 as abnormal case.

- **Impact of Event Scale – Revised (IES-R)** is a 22-item self-report measure that assesses subjective distress caused by traumatic events. Respondents are asked to identify a specific stressful life event and then indicate how much they were distressed or bothered during the past seven days by each "difficulty" listed. Items are rated on a 5-point scale ranging from 0 ("not at all") to 4 ("extremely"). The IES-R yields a total score (ranging from 0 to 88) and subscale scores can also be calculated for the Intrusion, Avoidance, and Hyperarousal subscales.

- **Activities of Daily Living** (**ADL)** (Katz, 1970) is an index to assess functional status of basic activities of independent living. There are six functions measured, bathing, dressing, toileting, transferring, continence and feeding. Summary scores range from 0 (low function, severe functional impairment) to 6 (full function). ADL will be assessed as perceived pre-COVID and current.

- **Instrumental Activities of Daily Living** (**IADL)** (Lawton, 1969) is an instrument to access independent living skills. These skills are considered more complex than the basic activities measured by the Katz ADL scale. There are eight categories, ability to use a telephone, shopping, food preparation, housekeeping, laundry, mode of transportation, responsibility for own medications, ability to handle finances. Summary scores range from 0 (low function, dependent) to 8 (high function, independent).

- **Pittsburgh Sleep Quality Index (PSQI)** is a self-rated questionnaire which assesses sleep quality and disturbances over a 1 month-time interval. Minimum Score = 0 (better); Maximum Score = 21 (worse). Interpretation: Total $\leq$ 5 associated with good sleep quality. Total > 5 associated with poor sleep quality. PSQI will be assessed as perceived pre-COVID and current.

- **Automated Self-Administered 24 hour dietary recall (ASA-24)** will be used to record two 24 hour dietary periods.

- **Multidimensional Fatigue Symptom Inventory (MFSI)** will also be used to measure perceptual fatigue. Fatigue symptoms will be measured using the 30-item

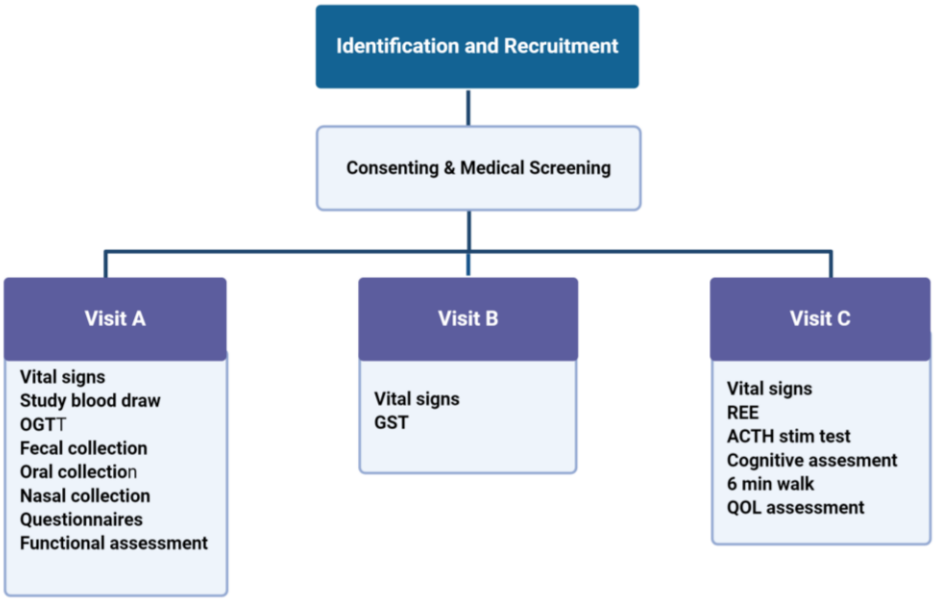
Multidimensional Fatigue Symptom Inventory – Short Form, a validated measure that yields one overall score of total fatigue (range -24-96, with higher scores indicating more fatigue) and five subscales (general, physical, emotional, mental, vigor). With the exception of the vigor subscale, higher scores indicate greater fatigue.

- **Assessment of Growth Hormone Deficiency in Adults (QoL-AGHDA)** Symptoms of growth hormone deficiency will be measured in subjects using the QoL-AGHDA. This 25-item questionnaire measures specific symptoms associated with growth hormone deficiency, with a score range of 0 to 25, with a higher score indicating worse symptoms.

- **The Beck Depression Inventory-II (BDI-II)** is one of the most widely used self-report inventories to assess depressive symptom severity in adolescent and adult populations (clinical and non-clinical). The BDI-II is comprised of 21 individual items reflecting specific cognitive, affective, and physical symptoms of depression. Each item includes four statements that vary in the description of symptom of severity. Scores range from 0 to 3, with a score of "3" indicating a severe symptoms and a score of "0" indicating an absence of concern with that particular aspect of depressive symptomology. The total score is the sum of all endorsed statements. If more than one statement from a given item is chosen by the respondent, only the statement of greatest severity is scored. The maximum total score is 63. The BDI-II Manual designates the following raw score classifications depression severity: ≤13 = *minimal*; 14-19 = *mild*; 20-28 = *moderate*; ≥ 29 = *severe*.

- **Gastrointestinal Symptom Rating Scale (GSRS)**. Gastrointestinal symptoms will be monitored using the 15-item GSRS. Each question has a seven-point Likert-type scale where 1 represents absence of symptoms and 7 represents very troublesome symptoms.

PASC Subjects



Interested subjects with a previous diagnosis of COVID will undergo a phone pre-screen including the Brief Fatigue Inventory (BFI) questions 1-3. If they score ≥ 3 on any BFI questions 1-3, and are interested in participating in the study, they will be scheduled for a formal consenting and medical screening at the UTMB Clinical Research Center (CRC). Subjects will report to the UTMB Institute for Translational Sciences (ITS) Clinical Research Center (CRC) for the medical screening and then for three separate study visits (Visits A, B and C). The study visits will consist of measurement of glucose tolerance, growth hormone and cortisol production, resting energy expenditure, functional testing including hand grip strength, gait measurements and 6 minute walk test, fatigue measurements, cognitive function assessment and questionnaires of health status, mood and quality of life, diet, sleep, and gastrointestinal health. Blood sampling for measurement

of amino acid levels, hormones, and metabolites will be drawn. In addition, fecal and oral samples will be collected for analysis of the GI and oral microbiomes. Visits A, B and C will be at least 7 days apart. All in person visits we will follow UTMB in-person procedures for safety.

Screening Procedures: All subjects consenting will undergo the following procedures. Screenings will take place at the UTMB ITS Clinical Research Center (ITS-CRC).

- Vital signs. Vital signs will be collected at screening. This will include height, weight, blood pressure, pulse, respirations, and temperature.

- Urine Screening Labs. Urine will be collected at screening for the following labs. The labs will be sent to the UTMB clinical laboratory for analysis and entered into the UTMB medical chart.

    - Urinalysis

- Urine Pregnancy Test. Urine will be collected from female subjects for a urine pregnancy test to be performed at the UTMB ITS-CRC as point of care testing (POCT). Women who are post-menopausal will be exempt from this test.

- COVID test. A PCR test using a nasal swab will be used to check ensure that the subject is negative for active COVID virus before beginning study. Subjects with negative PCR COVID tests within 2 weeks of screening date will not be retested.

- Screening Blood Labs. Subjects will have the following labs drawn for screening. Approximately 20 ml of blood will be drawn for screening labs listed below using a butterfly needle from a peripheral vein in the arm. The labs will be sent to the UTMB clinical laboratory for analysis and entered into the subjects UTMB medical chart:

    - Complete blood count with differential (CBC w/ diff)
    - Comprehensive metabolic panel (CMP)
    - Lipid Panel
    - Hepatitis B surface antigen
    - Hepatitis C virus antibody
    - HIV screen

- History and Physical. Subjects will undergo a medical history and physical examination by a study nurse, medical student or physician. Recent (within 3 months) clinical history and physical may be used in place.

Study Procedures:

Visit A

- Vital signs. Vital signs will be collected. This will include height, weight, blood pressure, pulse, respirations, and temperature. Vitals will be collected both sitting and standing.

- Urine. A clean catch urine will be obtained. Urine will be frozen at -80C and used to measure proteinuria, urinary microbiome signatures and renal function.

- Phlebotomy. Subjects will have a catheter placed into a peripheral vein in their arm (0.9% sodium chloride set at TKO) for the oral glucose tolerance test (OGTT). The IV will be discontinued when the OGTT is complete.

- Blood draw. Up to 20 ml of blood (2-serum separator top (5ml); 1-lithium heparin (6ml); 1-K-EDTA (4ml)) will be collected from fasted (at least 10 hours) subjects for analysis of hormones, amino acids and metabolites.

  An additional 20 ml of blood will be collected for the following labs to be sent to the UTMB clinical laboratory for analysis (all labs run at UTMB will be documented in the subject's UTMB medical chart) An additional 3.5 ml blood will be collected and frozen as a back up in case of a problem with labs sent to ARUP.:
  - Insulin-like Growth Factor 1 (IGF-1)
  - Follicle Stimulating Hormone (FSH)
  - Sex Hormone Binding Globulin (SHBG)
  - Total Testosterone
  - Free Testosterone
  - Prolactin
  - Thyroid Stimulating Hormone (TSH)
  - Free T4
  - High Sensitivity CRP
  - Vitamin B12
  - Vitamin D

- Oral Glucose Tolerance Test (OGTT). Glucose tolerance will be determined using a standard OGTT and a breath test. After an overnight fast (water allowed), an antecubital venous IV is placed for the collection of blood samples. During the baseline blood collection (~5 ml), a simultaneous breath sample is collected by having the subjects breathe into a breath collection bag fitted with one way valves. Following the baseline sample collection, a drink containing 75 g glucose and 150 mg U-^{13}C6-glucose is administered and consumed within 1 minute. From this point (t=0 minutes) blood (~5 ml) and breath samples are collected at 30 min, 60 min, 90 min, 120 min. For the collection of breath samples, the subjects are instructed to breath normally, hold their breath for 3 seconds, and exhale completely into the provided collection bags. Subjects remain at rest throughout the OGTT. Blood samples collected at specified time point will be analyzed for glucose and insulin at the UTMB clinical lab and will be documented in the subject's UTMB medical chart.

  The ratios of $^{13}CO_2$ to $^{12}CO_2$ in single breath samples are measured using a UBiT-IR300 infrared spectrophotometer (Otsuka Electronics Co., Ltd, Hirakata, Osaka, CV ≤ 1.0 %). All breath results are calculated as per mille (‰) change of $^{13}CO_2$ abundance from the baseline breath sample and expressed as ‰ delta over baseline (‰DOB).

- GI microbiome. The GI microbiome will be assessed using a self-collected fecal sample. Subjects will provide a fecal sample as described below. Samples will be stored at room temperature until transferred to UTMB (on/+ 5 days after collection). Subjects will be given a tutorial on how to properly collect the fecal sample. They will be given a handout from the manufacturer of the

collection kit with step by step instructions. They will also be shown a video of the collection process supplied by the kit manufacturer.

https://www.youtube.com/watch?v=ytr_hmJdHqM&feature=youtu.be&rel=0

Fecal specimens will be collected using the OMNIgene-Gut® (OMR-200) collection kit from DNA genotek (please see product inserts attached as pdf files). To assist in collection, the additional toilet accessory will be employed where appropriate (OM-AC1). The manufacturer's instructions will guide sample collection. Briefly, fecal material will be collected by catch on a kit-provided tissue or toilet hat that has been wiped with a disinfecting wipe prior to use. The study participant's fecal material will be transferred using the kit included spatula into the yellow tube top of a collection tube with the purple cap unscrewed. The tube will then be re-capped tightly and shaken for a minimum of 30 seconds to mix feces with kit included stabilization liquid. Storage recommendations as per manufacturer allow up to 60 days at room temperature or up to several months at -20°C or -80°C in the kit provided collection tube. Samples will be labeled with the subject's unique code and stored at room temperature until transported to University of Texas Medical Branch (UTMB). Samples will be processed and analyzed by qPCR as described below.

Nucleic acids will be extracted from batched fecal specimens using our optimized workflow that includes use of the DNeasy PowerMicrobiome kit (2600-50-1, Qiagen). If necessary, samples will be diluted/mixed 1:1 with sterile PBS to yield a pipettable liquid slurry. For each sample, 100 µl of the fecal liquid fraction will be mixed with 200 µl of kit-provided lysis buffer into a commercially available tube containing 0.1 mm glass beads (2600-50-BT, Qiagen). Samples will then be heated at 55°C for 30 minutes. Subsequently, samples will be homogenized for 5 minutes at 30 Hz using a Tissue Lyser instrument (Qiagen). Samples then will be centrifuged at 13,000 x g for 1 minute at room temperature. The liquid fraction will then be placed into a sterile microtube along with 50 µl of IRS solution (2600-50-2, Qiagen) and incubated at 4°C for 5 minutes. The sample will then be centrifuged as before and the liquid fraction taken for extraction of DNA using a MagNAPure 96 automated nucleic acid extraction platform (Roche). Purified DNA will be used for next-generation sequencing and qPCR analyses to create molecular profiles for each microbiome. Residual DNA will be archived at -20°C.

An additional collection will be performed to collect viable fecal material. Fecal samples will be collected using the spatula included in the OMNIgene-Gut kit used in the first collection. Briefly, after the participant will transfer fecal material to the yellow top of the collection tube for the first collection, then they will scoop another small amount of fecal material with the same spatula and transfer it to a prepared cryogenic freezer tube containing sterile PBS (without Ca/Mg) and 10% sterile glycerol. The tube will be closed and shaken for a minimum of 30 seconds to mix with the stabilization liquid. Samples will be labeled with the subject's unique code and stored at 4°C until transported to University of Texas Medical Branch. Samples will then be frozen at -80°C and stored until the molecular analysis is complete.

After initial analysis, if the sample is considered useful (representative of the average PASC subject), it may be thawed, diluted in saline and used either to mix in a mathematical ratio to create a synthetic community or to be directly transplanted into germ free mice. If the sample is considered not useful (not representative of the average PASC subject) for fecal microbiome transplant into germ free mice, the sample will be destroyed.

- Oral Microbiome. The oral microbiome will be assessed using saliva samples. Subjects will provide saliva samples as described below. Subjects will collect saliva samples at home. Samples will be stored at room temperature until transferred to UTMB (on/+ 10 days after collection). Subjects will be given a tutorial on how to properly collect the saliva sample. They will be provided a handout from the manufacturer of the collection kit with step by step instructions. They will also be directed to watch a video of the collection process supplied by the kit manufacturer.

  https://www.youtube.com/watch?v=N5K6LXmasK8&feature=youtu.be

  Saliva specimens will be collected using the Oragene-Discover® (OGR-500) collection kit from DNAgenotek (please see product inserts attached as pdf files). The manufacturer's instructions will guide sample collection. Briefly, saliva will be collected by catch in a funnel that is attached the collection tube. The subject will spit into the funnel repeatedly until the saliva reaches the "fill line" marked on the tube, which is 2ml. The tube will then be re-capped tightly. Once the top has clicked, the funnel will be unscrewed from the collection tube. The provided collection tube cap will be tightly secured and the tube will be shaken for a minimum of 5 seconds to mix saliva with kit included stabilization liquid. Storage recommendations as per manufacturer allow up to 5 years at room temperature or longer at -20°C or -80°C in the kit provided collection tube. Samples will be labeled with the subject's unique code and stored at room temperature until transported to University of Texas Medical Branch (UTMB).

  During the study visit, an oral swab will be performed by research staff or by the subject under direction of the research staff. The sterile calcium alginate swab will be swiped 5 times in each location listed: right cheek, left cheek, on the top of the tongue, under the tongue, on the roof of the mouth and along the gum lines. The swab will be immediately placed in a tube containing MagNAPure lysis buffer. The shaft of the swab will be snapped off and discarded leaving the head of the swab immersed in the lysis solution. The tube cap will be tightly secured and the tube will be shaken for a minimum of 5 seconds to saturate the head of the swab with the lysis buffer releasing the bacterial lysate into the fluid. Samples will be stored at -80C for subsequent batch DNA extraction on a MagNAPure96 followed by microbiome profiling by NGS and/or qPCR. Samples will be labeled with the subject's unique code.

- Nasal Microbiome. During the study visit, a nasal swab will be performed by research staff or by the subject under direction of the research staff. The sterile calcium alginate swab will be inserted

into the left nare until resistance is met, rotated 5 times and then moved to the right nare and rotated 5 times. The swab will be immediately placed in a tube containing MagNAPure lysis buffer. The shaft of the swab will be snapped off and discarded leaving the head of the swab immersed in the lysis solution. The tube cap will be tightly secured and the tube will be shaken for a minimum of 5 seconds to saturate the head of the swab with the lysis buffer releasing the bacterial lysate into the fluid. Samples will be stored at -80C for subsequent batch DNA extraction on a MagNAPure96 followed by microbiome profiling by NGS and/or qPCR. Samples will be labeled with the subject's unique code.

- Vaginal Microbiome (women only). During the study visit, a vaginal swab will be self-collected by the subject under direction of the research staff. The sterile calcium alginate swab will be inserted into the mid-vaginal area and rotated for at least 20 seconds. The swab will be immediately placed in a tube containing PBS buffer. The shaft of the swab will be snapped off and discarded leaving the head of the swab immersed in the solution. The tube cap will be tightly secured and the tube will be shaken for a minimum of 5 seconds to saturate the head of the swab with the buffer. Samples will be placed on wet ice and processed sterilely to create aliquots for microbiome evaluations. Samples will be labeled with the subject's unique code. This will be an optional procedure.

- Oral Photograph. A photograph will be taken of the upper and lower mouth and used to assess for the presence of periodontal disease and general oral health by a licensed dentist, who will be IRB approved.

- Post-COVID Recovery Clinic (PCRC) Questionnaires. A packet containing the PCRC battery of assessments that is currently being used in all PCRC participants will be provided to participants at Visit A. They may fill the packet of information at Visit A or take home and bring back the completed packet to their Visit B appointment. Questionnaires include: EQ-5D, MOS-SF-36 Version 2, Hospital Anxiety and Depression Scale (HADS), Impact of Events Scale (IES-R), Activities of Daily Living ADL (pre-COVID, current), Instrumental Activities of Daily Living IADL (current), Pittsburgh Sleep Quality Index (PSQI, pre-COVID, current), and 2 days of 24 hour dietary recalls (ASA-24).

    - **EuroQOL-5 Dimensions (EQ-5D)** is a 5 item assessment of health status measuring mobility, self-care, usual activity, discomfort or pain and depression or anxiety.

    - **Medical Outcome Study - Short Form 36 (MOS-SF-36)** is a questionnaire developed by RAND Health to measure patient self-reported quality of life. Eight subscales are measured including physical function, role limitations due to physical health, role limitations due to emotional problems, energy/fatigue, emotional wellbeing, social functioning, pain and general health. Scores for each subscale range from 0 - 100, with 100 being better perceived health and 0 being worst perceived health.

    - **Hospital Anxiety and Depression Scale (HADS)** is a 14 item assessment which includes 7 questions for anxiety and 7 questions for depression, which are summed separately for a total score for each. HADS classifies scores 0-7 as normal; 8-10 as borderline abnormal (borderline case); 11-21 as abnormal case.

- **Impact of Event Scale – Revised (IES-R)** is a 22-item self-report measure that assesses subjective distress caused by traumatic events. Respondents are asked to identify a specific stressful life event and then indicate how much they were distressed or bothered during the past seven days by each "difficulty" listed. Items are rated on a 5-point scale ranging from 0 ("not at all") to 4 ("extremely"). The IES-R yields a total score (ranging from 0 to 88) and subscale scores can also be calculated for the Intrusion, Avoidance, and Hyperarousal subscales.

- **Activities of Daily Living** (**ADL)** (Katz, 1970) is an index to assess functional status of basic activities of independent living. There are six functions measured, bathing, dressing, toileting, transferring, continence and feeding. Summary scores range from 0 (low function, severe functional impairment) to 6 (full function). ADL will be assessed as perceived pre-COVID and current.

- **Instrumental Activities of Daily Living** (**IADL)** (Lawton, 1969) is an instrument to access independent living skills. These skills are considered more complex than the basic activities measured by the Katz ADL scale. There are eight categories, ability to use a telephone, shopping, food preparation, housekeeping, laundry, mode of transportation, responsibility for own medications, ability to handle finances. Summary scores range from 0 (low function, dependent) to 8 (high function, independent).

- **Pittsburgh Sleep Quality Index (PSQI)** is a self-rated questionnaire which assesses sleep quality and disturbances over a 1 month-time interval. Minimum Score = 0 (better); Maximum Score = 21 (worse). Interpretation: Total $\leq$ 5 associated with good sleep quality. Total > 5 associated with poor sleep quality. PSQI will be assessed as perceived pre-COVID and current.

- **Automated Self-Administered 24 hour dietary recall (ASA-24)** will be used to record two 24 hour dietary periods.

- **Gastrointestinal Symptom Rating Scale (GSRS)**. Gastrointestinal symptoms will be monitored using the 15-item GSRS. Each question has a seven-point Likert-type scale where 1 represents absence of symptoms and 7 represents very troublesome symptoms.

- Functional assessments with wearable sensors. Functional capacity measures will be collected in (1) a standard laboratory setting and (2) in a simulated home setting (nearby empty room available for testing). First, using a Zeno gait mat and instrumented insoles we will collect gait capacity metrics during functional assessment tests. These tests will include the Short Physical Performance Battery, Timed Up and Go, and 2 Minute Step Test. Next participants will be given up to 30 minutes to rest. Participants will complete the same battery of assessments at the simulated home setting while receiving direction and observation via videoconference. Questionnaires will be provided to ask participants about the acceptability, usability, and technology preferences after completion.

- Handgrip Strength. Hand grip will be measured using a digital dynamometer (Jamar) three times on each hand. The maximal value for each hand will be recorded.

Visit B

- Vital signs. Vital signs will be collected. This will include height, weight, blood pressure, pulse, respirations, and temperature.

- Phlebotomy. Subjects will have a catheter placed into a peripheral vein in their arm (0.9% sodium chloride set at TKO) for the cortisol stimulation test. The IV will be discontinued when the cortisol stimulation test is complete.

- Glucagon stimulation test. Subjects will have a glucagon stimulation test. After the IV is placed, 3.5 ml of blood will be collected for the baseline (time: 0 minutes) to test for levels of human growth hormone. 1 mg glucagon (for subjects over 90 kg, 1.5 mg glucagon) will be injected IM in the deltoid muscle of the subject. Additional blood samples (3.5 mL) will be collected to test for levels of human growth hormone at time points: 90 minutes, 120 minutes, 150 minutes, and 180 minutes. All blood samples will be sent to UTMB clinical laboratory for analysis and entered into subjects UTMB medical chart.

Visit C

- Vital signs. Vital signs will be collected. This will include height, weight, blood pressure, pulse, respirations, and temperature.

- Indirect calorimetry for Resting Energy Expenditure (REE). Expired gases will be collected and analyzed for O_2 and CO_2 concentrations for the determination of energy expenditure and substrate oxidation in subjects using the ITS-CRC indirect calorimetry system, Vmax Encore, Carefusion. The subject will be asked to lie down on a bed and the ventilated canopy hood will be placed over their head and shoulders. The room will be darkened and external noises will be eliminated, as much as possible. The subject will be asked to relax and remain awake but as still as possible for the testing period of 30 minutes.

- ATCH (Cortrosyn) stimulation test. Subjects will have an ATCH stimulation test. 3.5 ml of blood will be collected using a butterfly needle from a peripheral vein in the arm for the baseline (time: 0 minutes) and analyzed for levels of cortisol. 0.25 mg Cortrosyn (1 vial) will be injected IM in the deltoid muscle of the subject. And additional blood samples (3.5 mL) will be collected at 30 and 60 minutes after the injection to test for levels of cortisol. All blood samples will be sent to UTMB clinical laboratory for analysis and entered into subjects UTMB medical chart.

- Phlebotomy. Subjects will have a catheter placed into a peripheral vein in their arm (0.9% sodium chloride set at TKO) for the ATCH stimulation test. The IV will be discontinued when testing is complete.

- Cognitive Assessment. Subjects will undergo a cognitive assessment.

  Montreal Cognitive Assessment (MoCA). The Montreal Cognitive Assessment (MoCA) is a rapid assessment of cognition. The MoCA consists of 9 questions with the following subcategories: visuospatial/executive, naming, memory, language, abstraction, delayed

recall and orientation. The MoCA has been used extensively to detect cognitive impairment in many conditions, including head trauma. Version 8.1 will be used.

- Modified 6-minute walk. Walking performance will be assessed in subjects during 6 minutes of walking in long corridor hallways. This is a standard test of walking performance that has been validated in similar studies. Subjects will be asked to walk at a 100% effort. Distance traveled for each 2-minute category will be recorded, as well as total distance.

- Questionnaires. Questionnaires will be completed to assess perceptual fatigue, symptoms associated with growth hormone deficiency, depression, and gastrointestinal symptoms.

    - **Multidimensional Fatigue Symptom Inventory (MFSI)** will also be used to measure perceptual fatigue. Fatigue symptoms will be measured using the 30-item Multidimensional Fatigue Symptom Inventory – Short Form, a validated measure that yields one overall score of total fatigue (range -24-96, with higher scores indicating more fatigue) and five subscales (general, physical, emotional, mental, vigor). With the exception of the vigor subscale, higher scores indicate greater fatigue.

    - **Assessment of Growth Hormone Deficiency in Adults (QoL-AGHDA)** Symptoms of growth hormone deficiency will be measured in subjects using the QoL-AGHDA. This 25-item questionnaire measures specific symptoms associated with growth hormone deficiency, with a score range of 0 to 25, with a higher score indicating worse symptoms.

    - **The Beck Depression Inventory-II (BDI-II)** is one of the most widely used self-report inventories to assess depressive symptom severity in adolescent and adult populations (clinical and non-clinical). The BDI-II is comprised of 21 individual items reflecting specific cognitive, affective, and physical symptoms of depression. Each item includes four statements that vary in the description of symptom of severity. Scores range from 0 to 3, with a score of "3" indicating a severe symptoms and a score of "0" indicating an absence of concern with that particular aspect of depressive symptomology. The total score is the sum of all endorsed statements. If more than one statement from a given item is chosen by the respondent, only the statement of greatest severity is scored. The maximum total score is 63. The BDI-II Manual designates the following raw score classifications depression severity: ≤13 = *minimal*; 14-19 = *mild*; 20-28 = *moderate*; ≥ 29 = *severe*.

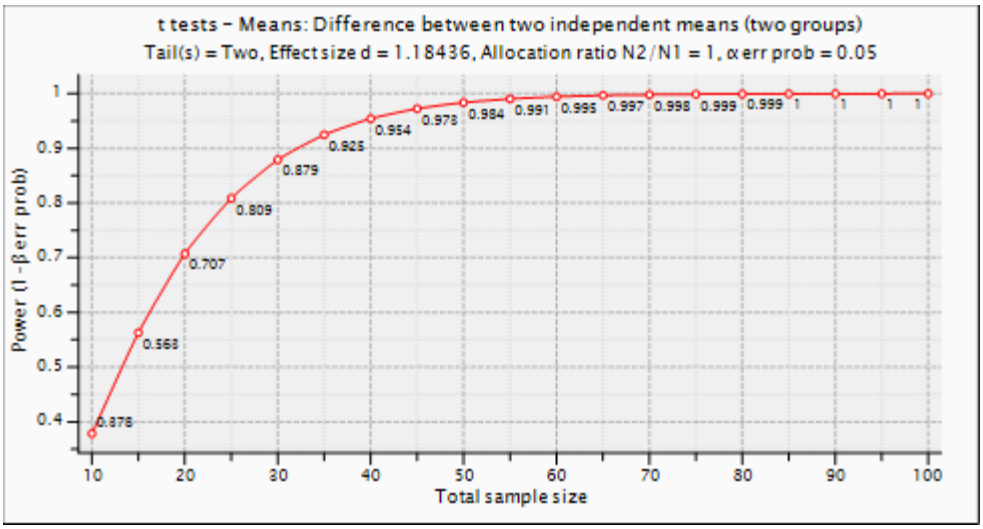
Statistical Considerations

In this observational study we intend to compare the microbiome community structures from the GI tract (fecal microbiome), oral, vaginal, and nasal mucosa sites in COVID long-haulers (subjects that meet our criteria for fatigue, altered cognition and are post-COVID-19) to community structures from our in house data as well as those published by others including the human microbiome project consortium. The observational hypothesis is that PASC subjects may have notable differences from normal subjects associated with the PASC sequelae described above. The study design is based on our past experiences with brain injury associated fatigue and altered cognition (BIAFAC) syndrome leading us to make practical group size selections of 32 symptomatic PASC patients. The design will include equal numbers of males and females (n=16 each). Because no publications are currently available describing changes to the microbiome in PASC patients, we are unable to complete specific power calculations. However, based on our previous clinical studies of BIAFAC patients with similar symptom presentation, we anticipate that

we would need 6 subjects per group to have an 80% probability of detecting significant differences in at least one of the previously identified BIAFAC organisms (two-sided, a=0.05). With the proposed subject numbers (n=32 completed subjects) we believe we will generate reasonable preliminary findings to identify differences in microbiome communities related to detection and/or abundance of specific organisms associated with specific PASC symptoms.

For quantifiable outcomes such as abundances of individual species in the GI microbiome and symptom related outcomes, a standard repeated measure ANOVA model will be used. When possible, age and other covariates will be brought into the model to account for individual variability and group differences. Significance tests will be performed at the alpha=0.05 level, and multiple testing corrections will be used to account for potential Type I error rate inflation. Nonparametric, generalized linear, and transformed response models will be considered as necessary to alter the model to account for deviations from standard assumptions (normality, linearity, etc.). Current data support statistical outcomes being established with group sizes similar or slightly larger than our previous BIAFAC studies.

The graph below is based on group difference in *Alistipes onderdonkii* that was associated with BIAFAC as an example of the data analysis for this study. Power calculations were performed using G*Power (two-groups, two-sided, a=0.05, Version 3.1.9.2, Germany). Effect size in this case was 1.184. Below the graph is a list of organisms we identified previously that we used to conduct past power analyses for similar studies. Please note that the intestinal and oral flora include an incredibly diverse system of organisms and at this time it is difficult to accurately predict which organisms will be included in potential microbial shifts. Therefore, additional organisms will be included in the microbiome comparisons between PASC patients and naïve control subjects. Further power analyses will be conducted using the data collected during this investigation.



Target	n = 20 (10/group)	n=30	n=40	n= 50 (25/group)
Alistipes onderdonkii	70.7%	87.9%	95.4%	98.4%
Desulfovibrio	60.4%	79.3%	89.9%	95.4%
Prevotella spp	97.5%	99.8%	100.0%	100.0%
Akkermansia	64.8%	83.2%	92.6%	96.9%
Odoribacter	52.5%	71.5%	83.8%	91.2%

Ruminococcus bicirculans	57.2%	76.3%	87.7%	93.9%
Bacteroides vulgatus	65.6%	83.9%	93.1%	97.2%
Sutterella	53.0%	72.0%	84.2%	91.5%
Lachnoclostridium	30.8%	44.4%	56.4%	66.4%
Streptococcus salivarius	27.8%	40.2%	51.4%	61.2%
16s	6.8%	7.8%	8.8%	9.8%

Study Timeline

This is a 1 year project.

Protection of Human Subjects

Human Subjects

Human subject involvement and characteristics. We propose to study a total 32 subjects (16M/16F) with a history of COVID and PASC (males and females) and 32 subjects (16M/16F) with a history of COVID without development of PASC between the ages of 18 and 80. This COVID, non-PASC group has been requested by several recent reviewers based on recent published evidence that non-PASC COVID recoveries may associate with an altered fecal microbiome.

See Protection of Human Subjects - Recruitment Methods and Consenting Process for detailed information regarding recruitment and consenting.

COVID Non-PASC controls
Inclusion criteria

1. Male or female with a history of COVID with diagnosis confirmed by PCR test.
2. Minimum of 6 months since diagnosis of COVID by PCR test.
3. Ages 18 to 80 years.
4. Participant is willing and able to give informed consent for participation in the study.

Exclusion criteria

1. Current COVID infection.
2. Unable to walk unassisted.
3. Significant heart, liver, kidney, blood or respiratory disease as determined by Principal Investigator.
4. Uncontrolled diabetes mellitus.
5. Any history of a recently (12 months) diagnosed cancer other than a skin cancer (excluding melanoma).
6. Current alcohol or drug abuse.
7. History of psychosis.

8. Pregnancy or become pregnant during the trial.
9. Subjects who are being managed with narcotics will be excluded as the effects of central nervous system depressants may interfere with study test results.
10. Other medical condition or medication administration deemed exclusionary by the study investigators.

PASC subjects
Inclusion criteria

1. Male or female with a history of COVID with diagnosis confirmed by PCR test.
2. Minimum of 6 months since diagnosis of COVID by PCR test.
3. Ages 18 to 80 years.
4. Score of 3 or higher on any question 1-3 of the Brief Fatigue Inventory (BFI) questionnaire.
5. Participant is willing and able to give informed consent for participation in the study.

Exclusion criteria

1. Current COVID infection.
2. Unable to walk unassisted.
3. Significant heart, liver, kidney, blood or respiratory disease as determined by Principal Investigator.
4. Uncontrolled diabetes mellitus.
5. Any history of a recently (12 months) diagnosed cancer other than a skin cancer (excluding melanoma).
6. Current alcohol or drug abuse.
7. History of psychosis.
8. Pregnancy or become pregnant during the trial.
9. Subjects who are being managed with narcotics will be excluded as the effects of central nervous system depressants may interfere with study test results.
10. Other medical condition or medication administration deemed exclusionary by the study investigators.

Sources of materials. We may obtain blood, urine, fecal, saliva and questionnaire data from the subjects. All data/samples will be collected solely for the purpose of experimentation. The blood will be used for screening and to measure metabolites, amino acids, hormones and measures of inflammation. Fecal, nasal and saliva samples will be used for determination of the GI, nasal and oral microbiomes. Questionnaire data will be used to assess quality of life.

Recruitment Methods and Consenting Process

a) Potential subjects may be patients of the investigators.

b) 1. PASC subjects may be recruited from the UTMB post COVID clinic in one of the following ways:

- In the clinic – potential subjects may be given a flyer along with Fast Fact sheet explaining the screening and study procedures in bulleted points. Potential subjects can read over the information with their families and decide if they would like to

enroll. If interested, the potential subject will contact the study team for a phone pre-screening to go over eligibility requirements (pre-screening) for the study (see attached pre-screening sheet). If potential subject passes the pre-screening and is still interested, the potential subject will be asked to come to the ITS-CRC at a time that is mutually convenient for both the subject and the study team for consenting and medical screening.

- Contacted by a post COVID clinic physician or nurse practitioner that they may be eligible for a research study for PASC patients and that the study team will contact them in a few days to see if they are interested. Contact may be in person or through phone call, by letter (see attached letter) by postal mail or myChart. If interested, subjects will be sent the study Fast Fact sheet explaining the screening and study procedures in bulleted points. Potential subjects can read over the information with their families and decide if they would like to enroll. If interested, the potential subject will contact the study team for a phone pre-screening to go over eligibility requirements (pre-screening) for the study (see attached pre-screening sheet). If potential subject passes the pre-screening and is still interested, the potential subject will be asked to come to the ITS-CRC at a time that is mutually convenient for both the subject and the study team for consenting and medical screening.

2. COVID, non-PASC control subjects may be recruited by referral from UTMB clinicians and/or advertisement in both the UTMB daily announcements, researchmatch.org and word of mouth.

3. Subjects in both groups may also self-refer to the study from surrounding clinics or from clinicaltrials.gov, flyers or UTMB announcements.

c) All subjects will have privacy at each of their visits to the ITS-CRC. Consenting will take place at the ITS-CRC in a private room following the consent process checklist provided by the UTMB Office of Clinical Research. Subjects will be given a private room for each study visit.

d) To minimize the possibility of coercion or undue influence all subjects will be given a copy of the approved consent forms and encouraged to discuss with their families prior to meeting the coordinator for the consenting process.

e) A waiver of consent is requested for identification and prescreening of potential subjects.

f) Vulnerable populations will be excluded from this study.

Subject reimbursement for participation.

- Non-PASC control subjects will receive $25 for screening and $50 for their study visit. If they complete the whole protocol, they will be reimbursed $75 for time and travel. Payment will be completed through Clincard.
- PASC Subjects will receive $25 for screening and $50 for each of the three study visits. If they complete the entire protocol, they will be reimbursed $175 for time and travel. Payment will be completed through Clincard.

Potential Risks

The potential risks for subjects enrolled in this project will be the following:

Vital Signs. There are no known risks associated with this procedure.

Urine Collection. There are no known risks associated with this procedure.

Nasal Swab. Risks include pain and a chance for minor bleeding from the nose.

Phlebotomy. All patients will undergo periodic blood draws throughout the study. The risks of phlebotomy are pain, infection, bruising and introduction of blood borne pathogens. Aseptic technique will be used to minimize these risks.

Blood draws. No more than 120ml will be collected for the entire study.

Fasting. The risks of fasting include fatigue, headache, hypoglycemia and dehydration.

Oral Glucose Tolerance Testing (OGTT). Risks involved with the OGTT are related to phlebotomy. The risks of phlebotomy are pain and infection. Aseptic technique will be used to minimize these risks.

Oral Administration of Stable Isotope Labelled Glucose. There is no known risk to giving stable-isotopic tracers orally. They are frequently employed clinically and for research purposes in breath tests to measure various metabolic responses.

Fecal Sampling: There are no known risks associated with this procedure.

Saliva collection: There are no known risks associated with this procedure.

Vaginal Swab: There may be a risk of discomfort.

Oral Photograph: There are no known risks associated with this procedure.

Questionnaires: There is no known risk to completing the questionnaires listed in this study.

Functional Assessments: There is a small risk of falling during the functional assessments which could lead to injury. Additional risks may include muscle tightness, soreness, and/or fatigue.

Handgrip testing and 6-minute walk: The exercise tests may cause muscle soreness, cramps, dizziness, and possibly irregular heartbeats. The risks of the 6-minute walk include falling, elevated heart rate and elevated blood pressure. All subjects will be accompanied by a trained staff member.

Glucagon Stimulation Testing (GST): The known side effects of the glucagon stimulation test are headache and nausea and possible vomiting.

Indirect calorimetry for Resting Energy Expenditure (REE): There are no known risks involved with these measurements.

ATCH (Cortrosyn) Stimulation Testing: Side effects of cortrosyn include allergic reaction, rash, lightheadedness, fainting, high blood pressure, rapid or slow heartrate and swelling in the limbs. Bleeding, bruising or infection may occur at the injection site.

Cognitive Testing: There are no known risks associated with this procedure.

Confidentiality: Divulgation of personal sensitive data is a general risk of clinical investigations involving human subjects.

Adequacy of Protection Against Risks

Recruitment and informed consent. Subjects will be identified from the UTMB post COVID clinic, self-referred from surrounding post-COVID clinics, clinicaltrials.gov, flyers or UTMB announcements.

1. Potentially eligible subjects may be informed about the study either during clinic visits or by a message from a post-COVID physician.
    - In the clinic – potential subjects may be given a flyer along with Fast Fact sheet explaining the screening and study procedures in bulleted points. Potential subjects can read over the information with their families and decide if they would like to enroll. If interested, the potential subject will contact the study team for a phone pre-screening to go over eligibility requirements (pre-screening) for the study (see attached pre-screening sheet). If potential subject passes the pre-screening and is still interested, the potential subject will be asked to come to the ITS-CRC at a time that is mutually convenient for both the subject and the study team for consenting and medical screening.
    - Contacted by a post COVID clinic physician that they may be eligible for a research study for PASC patients (see attached letter) and that the study team will contact them in a few days to see if they are interested. Contact may be through postal mail or myChart. If interested, subjects will be sent the study Fast Fact sheet explaining the screening and study procedures in bulleted points. Potential subjects can read over the information with their families and decide if they would like to enroll. If interested, the potential subject will contact the study team for a phone pre-screening to go over eligibility requirements (pre-screening) for the study (see attached pre-screening sheet). If potential subject passes the pre-screening and is still interested, the potential subject will be asked to come to the ITS-CRC at a time that is mutually convenient for both the subject and the study team for consenting and medical screening.

2. Potentially eligible subjects may contact the study team directly after viewing an IRB approved flyer or fast facts sheet or after viewing the study on clinicaltrials.gov, flyers or UTMB announcements.

Consenting will take place at the ITS-CRC in a private room following the consent process checklist provided by the UTMB Office of Clinical Research.

Protections against risks. All key personnel have completed and passed the certification exam of the NIH-mandated course on protection of human subjects. The screening tests will allow us to exclude *a priori* subjects with potentially higher risk of developing complications. Randall Urban, MD will oversee the medical aspects of the experiments. Protections against risks for all patients will be the following:

Vital Signs. There are no known risks associated with this procedure.

Urine Collection. There are no known risks associated with this procedure.

Nasal Swab. Care will be taken to minimize pain and/or bleeding.

Phlebotomy. The use of aseptic technique will be used to minimize the risk of infection, but will not eliminate it completely. Some patients may experience a sudden drop in blood pressure in response to the placement of catheters. Symptoms may include lightheadedness, nausea and possibly vomiting. There are no long-term effects associated with this response. In our experience, symptoms occur in 3.3% of our healthy subjects. All patients experiencing this response will be examined by a physician and given the option of discontinuing the study. If the physician, PI, study investigators, and patient find no reason to stop the study, the patient will be allowed to continue.

Blood draws. No more than ~65 ml of blood will be drawn at any visit. This around 15% of a typical blood donation, and in adult individuals with normal blood count does not expose to the risk of anemia. Less than 120 ml will be drawn over the 1 month period of the study.

Fasting. All subjects will be made aware of the risks of overnight fasting. They will be advised to drink water the night before and the morning of their visits. All subjects will be fed a meal after the completion of their study visits.

Oral Glucose Tolerance Test (OGTT). The risk are associated with phlebotomy. Care will be taken to minimize the risk of infection, but will not eliminate it completely.

Oral Administration of Stable Isotope Labelled Glucose. There is no known risk to giving stable-isotopic tracers orally.

Fecal Sampling. There are no known risks associated with this procedure.

Saliva collection: There are no known risks associated with this procedure.

Vaginal Swab: A qualified member of the research team will fully explain the procedure for self-collecting the vaginal swab safely.

Oral Photograph: There are no known risks associated with this procedure.

Questionnaires. There are no known risks associated with this procedure.

Functional Assessments. A member of the research team will supervise all functional assessment measurements.

Hand grip and 6-minute walk. Risk of muscle soreness or cramps can be reduced or avoided with an adequate warm-up. The subjects will be accompanied by a qualified staff member at all times.

Glucagon Stimulation Test. Subjects will be made aware of the risks of the test.

Indirect calorimetry**.** There are no known risks associated with these measurements.

ATCH (Cortrosyn) Stimulation Testing. Subjects will be made aware of the risks of the test.

Cognitive Testing. There are no known risks associated with this procedure.

Confidentiality. Only the investigators, the study personnel, and the Medical Records department of the UTMB Hospital will have access to the subjects' personal information. All data containing the name of the patients will be locked in the PI office. To preserve confidentiality, immediately after enrollment each subject will be assigned a code by which each research sample will be identified for further analysis, thus avoiding identification by non-qualified individuals.

Potential Benefits of the Proposed Research to the Subjects and Others

No benefits are expected for the subjects. The knowledge collected from the present studies may be of benefit to society, as these studies will help develop the scientific basis for characterizing PASC.

Importance of Knowledge to be Gained

This study will help characterize the symptoms that persist in patients after COVID.

Data Safety and Monitoring Plan

Study Title: The Endocrine, Metabolic and Microbiome Influence on the Post-COVID Syndrome

Type of Research Data or Events to be Monitored:

Total subject accrual will be monitored as well as adverse events, protocol deviations, violations and unanticipated problems. The risk level associated with this study is estimated to be minimum.

Methods and Frequency of Analysis:

Plan for Monitoring and Safety Review:

a. Randall Urban, MD, the Principal Investigator is the individual responsible for monitoring the safety environment of the participants.

b. Thorough monitoring of the recruitment, enrollment, retention, informed consent process, adverse events, study procedures, and the evaluation of primary and secondary endpoints will be carried out.

c. Potential subjects will be informed of all procedures involved in the study. If the subject agrees to participate, written consent will be obtained.

d. Information will be reviewed on a weekly basis by the PI and the research team.

Plan for Data Management:

a. Study data collected at UTMB will be recorded on case report forms. To preserve confidentiality, immediately after enrollment each subject will be assigned a code by which each research sample will be identified for further analysis to avoid identification by non-qualified individuals. Dr. Urban, the Principal Investigator, is the individual responsible for storage of data. Recognizable personal subject information will be stored in Dr. Urban's laboratory in a locked cabinet.

b. Data will be reviewed on a weekly basis by the PI and the research team.

c. The conditions that would necessitate early termination of the study include withdrawal of consent.

d. The PI with the aid of the Co-Investigators and the Study Coordinator will perform aggregate analysis of data and adverse events.

Persons Responsible for Data Monitoring:

The ultimate responsibility for data and safety monitoring, and submitting reports of unanticipated problems, adverse events, protocol deviations and protocol violations rests with the Principal Investigator, Randall Urban, MD. Every effort will be undertaken to monitor and minimize the risks to subjects. Prior to obtaining informed consent, subjects will be encouraged to thoroughly read the informed consent form and ask questions regarding the outlined procedures and risks. To ensure ongoing protection of study subjects all procedures will be performed under the supervision of Randall Urban, M.D., or a study physician willing to provide backup medical coverage. All study physicians will be familiar with the study protocol.

Reporting Unanticipated Problems, Adverse Events, Protocol Deviations and Protocol Violations:

a. Dr. Urban, the Principal Investigator, is the individual responsible for monitoring and reporting the occurrence of unanticipated problems, adverse events, protocol deviations and protocol violations throughout the study.

b. Anticipated Adverse Events, as discussed above will be included in the consent form. All other anticipated adverse events will be addressed by the PI and study team. If an adverse event occurs Dr. Urban will be immediately notified and a note will be entered in to the subject's medical chart using the grading scale listed below.

c. Adverse Event Grading Scale (UTMB – CRC Scale):

0 = No Adverse Event or within normal limits

1 = Mild Severity: Transient laboratory test alterations; discomforts noted but no disruption of daily activities; no therapy, or only symptomatic therapy required.

2 = Moderate Severity: Laboratory test alterations indicating injury without long-term risk; discomfort sufficient to modify normal daily activity; specific therapy required (i.e., more than symptomatic).

3 = Serious Severity: Laboratory test indicating a serious health threat or permanent injury; incapacity, inability to work, inability to perform normal daily activity; hospitalization required or prolonged; emergency treatment required; life-threatening events; death.

The attribution scale assesses the relation of the event to the study procedures. The Principal Investigator will judge whether or not an adverse event is: 1) not related; 2) possibly related; 3) probably related; 4) definitely related to the study procedures and interventions.

All unanticipated, serious, fatal and/or life-threatening adverse events will be reported to the IRB within 24 hours of occurrence. The PI and UTMB Institutional Review Board (IRB) are responsible for determining whether modifications to the protocol and consent form are required. If a determination is made that participants are found to be exposed to excessive risks in relation to anticipated benefits, the study will be immediately suspended. Studies will not resume until modifications are made that are deemed to result in an acceptable risk/benefit ratio by the PI and IRB. Aggregate reports of adverse events will be prepared on an annual basis and forwarded to the IRB at annual review.

Stopping Rules:

The study would be stopped and re-evaluated if a subject had a loss of confidentiality.

Procedures and Time Frames for Communicating Outcomes:

Monitoring Reports will be prepared and forwarded to the IRB on an annual basis.

Precautions for Maintaining Data Integrity:

Careful monitoring of the recruitment, enrollment, retention, adverse events, and study procedures will help to protect the safety of study subjects, the quality of data, and the integrity of the study. As part of the safety plan for this study, the PI will review individual study subject records as needed to ensure that appropriate mechanisms to protect the safety of study participants are being followed, that protocol requirements are being adhered to, and that data is accurate, complete, and secure. Subject records include consent forms, case report forms, flow of data forms, laboratory specimen records, inclusion/exclusion forms, and/or adverse event logs, and medical charts. The PI meets with the study team once per week to discuss ongoing study status.

Records Retention

The investigator shall retain the records for 15 years.

Inclusion of Women and Minorities

Women and men will be included.

Minorities will be included in the proportion found in the general population (see Targeted Enrollment Table)

Targeted/Planned Enrollment Table

Study Title: The Endocrine, Metabolic and Microbiome Influence on the Post-COVID Syndrome

Total Planned Enrollment: Up to 120 subjects

Targeted/ Planned Enrollment: Number of Subjects			
Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino	10	10	20
Not Hispanic or Latino	50	50	100
Ethnic Category: Total of All Subjects *	60	6060	120

Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	10	10	20
White	50	50	100
Racial Categories: Total of All Subjects *	60	60	120

Inclusion of Children

Children will not be included in this study. The age range for inclusion is 18 to 80.

Appendix A

Dr. Wright has a dual appointment with Texas A&M University, Department of Health and Kinesiology and University of Texas Medical Branch at Galveston, Department of Internal Medicine.He will conduct the following study procedures at Texas A&M: data analysis, manuscript writing. No subjects will be enrolled at Texas A&M University. Texas A&M University will rely on UTMB IRB as the IRB of record for this study.