TUFTS MEDICAL CENTER/TUFTS UNIVERSITY RESEARCH PROTOCOL

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Study Title: Role of the gut microbiome and the serum metabolome on lean mass and physical function in older adults

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I. Research Plan (Including Statistical Analysis)

40 older adults (70-85y) will be recruited from the local Boston area based on their performance on the short physical performance battery (SPPB), a measure of lower extremity physical function that includes standing balance, a 4-m walk and chair stand tests[1]. The SPPB has been extensively validated as predictive of future disability risk and mortality in older adults[1]. High-functioning (HF; SPPB ≥ 11) and low-functioning (LF; SPPB ≤8)[2] groups will each contain 20 subjects (10 men, 10 women). Gut microbial composition is affected by age[3, 4]. Similarly, sex and BMI are associated with gut microbiome composition[5-7]. With the goal of minimizing gut microbial variability, I propose to recruit HF and LF subjects that are matched for age, sex and BMI but are different in terms of lean mass and physical function.

At baseline, subjects will provide a blood and stool sample that will be subjected to serum metabolomics and fecal sample 16S ribosomal RNA (16S rRNA) gene profiling, respectively. Serum metabolomics provides an analytical description of complex biological media by identifying a large number of small molecule metabolites in a given biological sample[8]. Fecal sample 16S rRNA gene profiling will be used to identify the bacterial relative abundance that is present within each taxonomic level in each fecal sample. Lean mass and physical function will be measured in all subjects. Associations between serum metabolites, fecal bacteria, and between fecal bacteria and serum metabolites with the differentiation of HF from LF, and that are associated with lean mass and specific measures of physical function will be determined. In addition, associations between gut bacteria with circulating markers of insulin resistance and endotoxemia will be identified. Overlapping associations between gut bacteria and serum metabolites with these outcomes is expected to provide an enhanced understanding about the role of gut bacteria on mechanisms that underlie the maintenance of lean mass and physical function in older adults. The causative role of gut bacteria on the maintenance of lean mass and physical function in older adults will be examined by transplanting fecal bacteria from older adults into germ-free mice.

After 1 month, to determine the reproducibility of the baseline gut bacterial associations with lean mass and physical function, study subjects will return to provide a fecal sample that will be subjected to 16S rRNA profiling. Lean mass and physical function will be measured, and associations between fecal bacteria that collectively differentiate HF from LF, and that are associated with lean mass and specific measures of physical function will be identified and compared with baseline findings.

Statistical power: Sample size estimates are based on gut bacterial associations with the GFI[9]. Means ± SD for *Bacteroides*/*Prevotella* of 26.3 ± 13.2 and 14.8 ± 15.1 in the low-frailty and high-frailty groups, respectively, yield an effect size of 0.81, a value that is sufficient to obtain 80% power at a type-I error probability of 0.05 with a study sample size of 40 subjects. To achieve this recruitment goal, up to150 subjects will sign the Preadmission ICF, with the goal of 40 participants completing the study.

Blood and Stool Collection: Following an overnight, 12-hour fast (beginning between 8PM-12AM the day before), eligible candidates will provide a blood sample during the initial visit (baseline) and 1-month study visits. At the screening visit, eligible subjects will be provided with a stool collection kit so that they may provide a stool sample within the 18-hour period that precedes their baseline and 1-month visits. Fasting is optional for providing the stool sample.

Measurement of insulin, glucose, lipopolysaccharide (LPS) and physical function: Serum measurement of insulin and glucose are used to calculate the homeostasis model assessment (HOMA-IR), a circulating index of insulin resistance[10]. LPS is a component of gram-negative bacterial cell walls that can appear in the blood by microbial translocation from the gastrointestinal tract[11], is a circulating marker of metabolic endotoxemia[12]. LPS induces insulin resistance[12], myotube atrophy and decreased muscle function *in vitro*[13, 14], and decreases muscle mass in rodents[15]. Insulin, glucose and LPS will be measured by the Nutrition Evaluation Laboratory (NEL) at the Jean Mayer HNRCA at Tufts University.

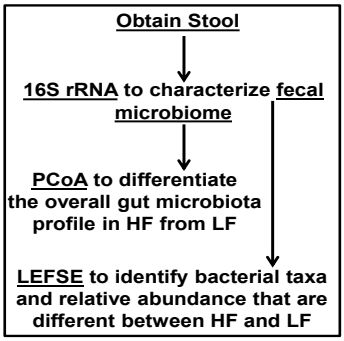
Measures of physical function including the SPPB, leg press one repetition maximum (1 RM) and 400-m will be performed by the NEPS laboratory at the Jean Mayer HNRCA at Tufts University[16]. The importance of measuring the SPPB, the leg press 1 RM and 400-m is illustrated by the finding that reduced performance in each of these measures is associated with increased disability[1, 17, 18] and an increased all-cause mortality risk[1, 18, 19].

Measurement of Dietary Intake: To account for the influence of dietary intake on the gut microbiome, three 24-hour dietary recalls will be collected from enrolled study subjects. Two of these recalls will be collected on the two days prior to the baseline visit. The final dietary recall will be performed on the day of the baseline visit, for a total of three 24-hour dietary recalls.

AIM 1: Identify associations between gut bacteria and circulating metabolites that collectively differentiate HF from LF, and are associated with lean mass and physical function in older adults. Dr. Lustgarten recently reported significant associations between circulating gut bacteria-related metabolites[20-22] with lean and skeletal muscle mass[23] and with measures of physical function[16], evidence that suggests a role for gut bacteria on the maintenance of these outcomes in older adults. To date, studies aimed at identification of associations between gut bacteria and circulating metabolites with lean mass have yet to be published, whereas two studies have reported associations between gut bacteria with function[9, 24]. However, the indices used to measure function were not specific for physical function, as questions about vision, hearing, cognition and psychosocial function were included[25-27]. Therefore, the objective of **AIM 1** is to characterize the association between fecal bacteria and circulating metabolites that collectively differentiate HF from LF, and that are associated with lean mass and physical function in older adults.

Identification of bacterial taxa present in fecal samples: Fecal sample 16S rRNA gene profiling will be used to identify the fecal bacteria relative abundance that is present within each taxonomic level. The Phoenix Laboratory at the Tufts University Medical Center[28] will extract fecal sample DNA, generate 16S rRNA amplicons, and assemble amplicon pools for high-throughput sequencing. The Tufts University Genomics Core Facility[29] will then use an Illumina MiSeq System (Illumina, San Diego, CA) to target the V4 region of the 16S rRNA gene. 16S rRNA sequences will be quality filtered, clustered into Operational Taxonomic Unit (OTUs) and further analyzed using QIIME (quantitative insights into microbial ecology)[30]. Use of 16S rRNA gene profiling allows for the determination of bacterial taxa other than *E. coli* and *Enterobacteriaceae* that may be different in HF when compared with LF older adults. For example, various bacterial species other than *E. coli* are capable of BCAA synthesis[31]. Furthermore, the BCAA catabolic metabolites 2-oxoisovalerate and 4-methyl-2-oxopentanoate are negatively associated with colonic *Lactobacillus spp*. abundance[21].

Bacterial taxa that differentiate HF from LF older adults: Principal co-ordinate analysis (PCoA) of the phylogeny based Unifrac distance metric[32], which are derived from OTUs[33] will be used to determine if the overall gut microbial profile in HF is significantly different when compared with LF older adults. The analysis of similarities function in the statistical software package PRIMER 6 (PRIMER-E Ltd., Lutton, UK[34]) will be used to determine between-group statistical significance ($p \leq 0.05$). To identify bacterial taxa and relative abundance that are significantly different in HF when compared with LF older adults, linear discriminant analysis effect size (LEFSE)[35] will be used. LEFSE is an algorithm for high-dimensional biomarker discovery and explanation that identifies bacterial taxa that characterize the difference between two or more biological conditions.



Test the causative role of human gut bacteria on the maintenance of lean mass and physical function: Although identification of gut bacteria that are associated with lean mass and physical function is an important observation, transplanting intact uncultured human fecal microbiota into recipient germ-free mice permits the donors' communities to be replicated and the causative role of their microbiota on outcome measures to be

discerned. For example, transplantation of human gut microbiota from 4 female twins discordant for kwashiorkor (the condition caused by severe dietary protein deficiency) into germ-free mice resulted in 86-90% of species-level taxa and ~90% of the functions encoded by the donor's microbiome being found in recipient mice, thereby implicating the gut microbiota as a causal factor on kwashiorkor[36].

Transplantation of gut microbiota from a healthy human adult into germ-free mice resulted in all bacterial phyla, 92% of bacterial classes and 88% of genus-level taxa being detected in the feces of recipient germ-free mice[37]. Human gut bacteria-containing mice fed a high-fat western diet gained significantly more adiposity during the two weeks following transplantation than mice that consumed a low-fat, plant polysaccharide-rich diet[37], evidence that demonstrates a causative role for diet-induced increases in adiposity by human gut bacteria. Similarly, transplantation of human gut microbiota from 3 twins (2 female and 1 male) discordant for obesity into germ-free mice resulted in ~75% of family-level bacterial taxa and 99.7% of the functions encoded by the donor's microbiome being represented in recipient mice, thereby resulting in a greater increase in adiposity in mice colonized with fecal microbiota from obese, when compared with its lean co-twin[38].

Collectively, these studies demonstrate that transfer of gut bacteria from humans into germ-free mice is highly efficient, functions encoded by the transferred microbiome are maintained, and phenotypes are transmissible. The objective of **AIM 2** is to test the causative role of human gut bacteria on the maintenance of lean mass and physical function by transferring fecal microbiota from HF and LF older adults into germ-free mice.

Statistical Power: Germ-free mice colonized with fecal bacteria from obese humans gained significantly more adiposity than germ-free mice colonized with fecal bacteria from their lean co-twin (10.0% ± 5.0% compared with 0.1% ± 2.0%), a result that was significant 15 days after colonization[38]. Based on these data, the resulting effect size of 2.6 is sufficient to obtain 99% power at a type-I error probability of 0.05 with a sample size of 12 human subjects. Accordingly, with the goal of identifying a similar change in lean mass and physical function, I propose to transfer fecal bacteria from 12 older adults into germ-free mice.

III.2.a Colonization of germ-free mice with human gut bacteria from HF and LF older adults: Fecal samples from 6 HF (3 males, 3 females) and 6 LF (3 males, 3 females) older adults will be transferred into sex-matched (with the goal of minimizing sex-associated gut microbial variability[6, 7]) C57BL/6J germ-free mice for a duration of 5 weeks at the Germ-Free and Gnotobiotic Microbiology Core at the Harvard Digestive Disease Center (HDDC; Boston, MA). The transmissible effect of host phenotype by gut microbiota has been previously reported by transferring human fecal bacteria into C57BL/6J germ-free mice[36-38]. Furthermore, the homogenous genetic background provided by inbred C57BL/6J germ-free mice allows for a cleaner system to dissect signals from gut bacteria-host interactions, and improves experimental reproducibility, when compared with genetically heterogeneous GFM[39]. One fecal sample from each human donor will be transferred into 5 separate germ-free mice (based on the range of germ-free mice used in human fecal transfer experiments[36, 38]), resulting in 60 mice colonized with human gut microbiota. 5 germ-free mice will not be colonized with fecal bacteria from older adults (germ-free control group). All recipient mice will be fed *ad libitum* with a commercial, sterilized mouse chow[38]. Food consumption will be measured daily to ensure that changes in lean mass or physical function do not result because of differences in food intake.

III.2.b Measurement and analysis of the change in lean mass and physical function in mice colonized with human gut bacteria: Lean mass and physical function in mice colonized with human gut bacteria will be measured on day 0 (prior to colonization), 9, 18, 27 and 35 days post-colonization at the HDDC. Lean mass will be measured with use of quantitative magnetic resonance imaging (EchoMRI-3in1 instrument, Houston, TX)[38]. Muscle strength will be measured using a grip strength meter for mice (Technical and Scientific Equipment GmbH, Bad Homburg, Germany)[40]. Each mouse will firmly grab the pulling bar of the grip strength meter with both forepaws and then pulled gently backwards until it releases its grip. The peak force of each trial is considered the grip strength. A 1-liter beaker filled with water will be used as a swimming pool to test endurance capacity[41].

A weight (10% of body weight) will be attached to the tail of a mouse, which is then placed in the water. The amount of time that the mouse is able to maintain complete buoyancy will be recorded. The EchoMRI, grip strength meter and beaker/weight will be sterilized prior to use to ensure that germ-free conditions at the HDDC are maintained. Because Dr. Lustgarten has previously measured lean mass and physical function in mice[42, 43], he will perform these measurements at the HDDC.

Differences for the change in lean mass and physical function will be calculated relative to initial values. Statistical significance for the change in lean mass and physical function will be compared in mice colonized with fecal bacteria from HF or LF human donors and for germ-free mice that were not colonized with human gut bacteria with use of two-way ANOVA[38].

III.2.c Bacterial taxa identification and association with the change in lean mass and physical function in mice colonized with human gut bacteria: Fecal pellets will be obtained from 5 germ-free mice at baseline, which will not be colonized with human gut bacteria. Five weeks post-colonization with human gut bacteria, fecal pellets will be collected from 60 mice (30 each from the HF and LF donors) and from the 5 germ-free mice that did not receive human gut bacteria. 16S rRNA gene profiling on these 70 fecal pellets will be performed as described in **Section III.1.a**.

Associations between gut bacteria-containing principal components and individual bacterial taxa with the 35-day change in lean mass, mouse grip strength and endurance capacity will be identified as described in **Section III.1.b**. Supervised machine learning (random forest) will be used to predict whether a sample came from a mouse colonized with HF or LF donor microbiota[44].

The gut bacterial profile that is associated with the change in lean mass and physical function in mice colonized with HF or LF human fecal bacteria is expected to be similar to the gut bacterial profile that is associated with differentiation of HF from LF older adults. CIA will be used to identify associations between circulating metabolites with gut bacteria that collectively differentiate mice colonized with HF or LF gut bacteria.

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