Below is the "Research Strategy" portion of an R21 grant submission to the National Institutes of Health, part of a project with Rebecca Lee (Nursing), Joshua Gross (Biology), and Mao-Bing Tu (Environmental Engineering) (unfunded).

The completed application – including Specific Aims, letters of support from the Maple Knoll Retirement Living Community and the Vice President of Research, workplan, budget & justification, protection of human participants protocols, leadership plan, resource sharing plans, etc. – are available upon request.

RESEARCH STRATEGY

Background Factors contributing to loneliness among older adults are diverse, complex, and common [19]. These risk factors can arise from characteristics of the suffering individual, including a diminished sense of belonging, emotional/psychological distress, cognitive impairment, physical disability, disease, a perceived lack of social support, emotionally distant relationships, and a lack of mobility [20]. Vulnerability to loneliness can also arise from changing external circumstances, including lack of instrumental support (transportation, phones, internet access), a recent move to a new location, being unmarried, being a member of a minority group, infrequent contact with a social network, a lack of diversity in one's social network, and unavailability of social activities [21]. Several factors are especially common in older adults, such as retirement, wherein many older adults enjoy fewer work-related relationships and a diminished sense of purpose [22]. Deaths of loved ones and acquaintances leads to fewer relationships and a weakened social network [23]. In particular, a spousal death, a health crisis, and an "empty nest" place the aging population at particular risk of persistent loneliness [5]. Furthermore, as memory deficits develop, older individuals may experience social embarrassment and reduced executive functioning, which further diminishes motivation for social interaction [24]. Most recently, the COVID-19 global pandemic demonstrated that a natural or biological disaster can dramatically increase isolation of older adults, thus putting them at risk of increased loneliness [25]. Consequences of loneliness include higher morbidity and mortality, increased rates of dementia, more frequent/longer hospitalizations, falls, poor health practices, psychological distress, neglect and exploitation, and lower self-reported health and well-being [26,27]. Loneliness also places older adults at a higher risk of depression and anxiety, is associated with an unhealthy diet, and fewer visits to primary health care providers [28]. Additionally, loneliness can impact immune function leading to increased susceptibility to infection, disrupted sleep, and a higher risk of disability and cognitive decline [29]. As of 2017, the financial consequences of loneliness were estimated to cost the US government 6.7 billion dollars in annual health care expenses [30].

(a) ***SIGNIFICANCE*** Loneliness has long been recognized as an important public health concern, however a "universal treatment" for loneliness does not exist. This is explained, in part, by the large number of complicating societal, psychological, environmental, and biological factors, which are circumstance-specific and differ widely among individuals. These include, for example, the fact that between 1980 – 2010, there was a 40% increase in persons living alone, in largely solitary environments [31]. During the same period, social expectations changed such that more couples elected to not have children, thus decreasing level of social support. In the past ~30 years, social media has dramatically grown in popularity [32], but its "passive use" provokes feeling of detachment, inferiority, and envy, and related psychological conditions [33]. Moreover, internet use and accessibility to broadband varies with education level, geographical location, and physical ability which can lessen its positive impacts for under-resourced persons [34]. What is important is that individuals tend to be affected by these conditions differently. Loneliness is an irreducibly complex phenomena, a fact belied by its nearly universal presence across individuals, culture, and times [36][33][34].

A number of treatments for, as well as protective factors against, loneliness are known [35,36]. Of critical importance to older populations is a healthy social network [37], which buffers stress, increases access to resources, and stimulates "brain health" [38]. In particular, being around people with healthy habits reinforces healthy behaviors, improves access to health-related information, promotes better nutrition, and inspires more physical activity [39]. Despite a vast literature on these treatments and protective factors, none are *universally* effective. We believe this is due to the fact that loneliness cannot conform to a singular definition [40]. Rather, the experience of loneliness is etiologically complex and specific to each individual. Our overarching goal in this application, therefore, is not to understand the common forces that impact loneliness across the population (see Fig. 1A). *Rather, we seek to understand the personal, lived experience of loneliness in light of its heterogenous causal antecedents*. We propose that loneliness must be examined and treated as a dynamic, complex, and unique condition that can only truly be understood at the level of the individual through a multi-dimensional and transdisciplinary analysis of social, psychological, environmental, and biological factors (Fig. 1B).

(b) ***INNOVATION*** **(**1) A transdisciplinary approach to loneliness: The approach we propose here, to our knowledge, is the first to a use congruent mixed-methods methodology to evaluate the social, psychological, environmental, and biological factors influencing loneliness. This approach will allow us to characterize how these features interact in the life of an individual in a way that transcends traditional disciplinary boundaries. It

will therefore allow us to characterize the etiology of loneliness in a more precise and comprehensive way. The transdisciplinary approach will also allow for (2) A personalized, tailored approach to understanding loneliness: Our approach is motivated by the vast literature on loneliness, which will facilitate discriminating between aggregate and personalized measures of loneliness in the older adult population. The proposed work is novel in that it does not aim to define "universal" components of loneliness to develop generalized treatments. Rather, this work aims to understand the highly specific, highly contextualized components of loneliness. "Universal" approaches to loneliness are necessary when its etiology is considered only in broad contexts and along single dimensions. However, the full complexity of the trans-disciplinary approach introduces a sufficient number of heterogenous variables that we are better able to characterize loneliness at the individual level. This open the future possibility of highly tailored interventions. *To our knowledge, this is the first proposed approach seeking to understand the subjective experience of loneliness through a holistic examination of its social, psychological, and environmental context (Aim 1), as correlated with some of its biological markers (Aim 2)*.

(c) *APPROACH*

Aim 1. Examine individual psychosocial and environmental factors influencing loneliness in older adults. A comprehensive understanding of the psychosocial and environmental factors that influence loneliness in older adults is essential to designing effective interventions. In this study, we utilize narrative research techniques, alongside more traditional analyses of social network and the environment, to gain insight to the 'lived experience' of loneliness. As an individual makes their way through life, they have myriad experiences and interactions with their environment, the people in that environment, and with themselves. In order to structure these experiences and make sense of one's own and other's behavior, an individual repeatedly produces narrative descriptions that unify and ascribe meaning to the experiences. Narrative research, the study of how human beings experience the world, allows researchers to collect these stories [41]. These narratives can then be joined with more traditional analyses of social support networks and environmental conditions. Herein, these narratives will also be viewed through the lens of a potential biological changes to the microbiome (Aim 2) to gain further insight to this human condition. Social support networks refer to the social ties that bind an individual to their community and the characteristics of those ties [42]. Positive interaction with a social support network is associated with a wide range of health outcomes in older adults and reduces the risk for social isolation [43].

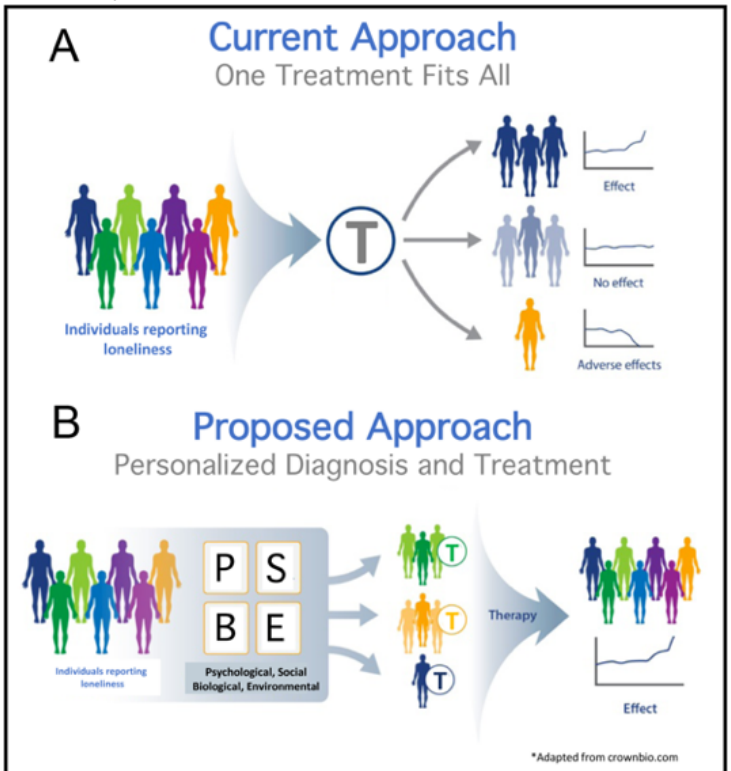

Figure 1. A novel, personalized approach to treatment of loneliness. Contemporary approaches to understanding and treating loneliness have advanced under the assumption that one treatment approach will address all individuals. In this proposal, we propose to develop an personalized approach to diagnosing loneliness in older adults, and developing more effective ways to mitigating this unique, negative subjective experience.

Experimental Methods

1.1. Completion of enrollment surveys: Older adults (n=60) will be enrolled into our study and complete the UCLA Loneliness Scale [11], the prevailing self-report instrument used in loneliness research to identify the degree of loneliness for an individual along a continuum. Based on their score, participants will be classified as either lonely or not lonely. Participants will also complete the Center for Epidemiologic Studies Depression Scale Revised (CESD-R), a screening test for depression and depressive disorder [cite]. As previously discussed, for older adults, loneliness serves as a strong predictor of depression. Finally, participants will complete a Demographic survey.

1.2. Completion of narrative interviews: We will pursue our long-term goal of developing tailored, targeted interventions by exploring the individual life stories of participants through narrative research. Each individual enrolled will participate in narrative interviewing. This qualitative inquiry strategy will reveal significant life experiences, including the experience of

loneliness, as described by participants. Participants will engage in extensive in-depth conversations with the researcher during which they narrate the story of their life, naturally highlighting those experiences that were significant to the storyteller.

1.3. Completion of social network and environmental analysis. In order to examine the social and environmental influences on loneliness in older adults, each participant will also complete the Lubben Social Network Scale and an Ecomap. The LSNS scale is designed to gauge social isolation in older adults by measuring the level of perceived social support received from family, friends and neighbors [44]. In addition, participants will also discuss their activities and environmental interactions, allowing the interviewer to complete an Ecomap for each individual. Ecomaps are a graphical representation that shows the social and community engagement and resources of individuals [45], which in turn will capture the diversity of economic backgrounds of individuals. This will allow us to evaluate the relationship between environmental resources, both social and physical, and the levels of loneliness experienced by older adults. Collectively, this information will inform the role of social and environmental elements in influencing loneliness and provide potential targets for future intervention. A) Sampling plan: We will identify participants through recruitment flyers that include a short description of our study, including contact information for those individuals interested in participating. These flyers will be posted in visible areas at our partner site, Maple Knoll Village, a retirement community in Greater Cincinnati, and in resident mailboxes. Recruitment flyers will also be distributed to older adults living outside the Maple Knoll campus, but who receive services as part of Maple Knoll community outreach. The study will further be communicated to residents at meetings/presentations at Maple Knolls. Researchers, alongside Maple Knoll staff, will present the study at regularly scheduled resident meetings. B) Recruitment and inclusion/exclusion criteria: Individuals will be recruited from the population of residents of the Independent Living housing at Maple Knoll and from older adults receiving outreach services from Maple Knoll, but who reside independently in the surrounding communities. Inclusion criteria for enrollment: a) age 65 – 85; b) can read and speak English; d) are willing and able to participate in two 60–80-minute face-to-face interviews; e) are willing and able to sign an informed consent document. Exclusion criteria include the following: a) the individual has a cognitive impairment; and b) are not able to speak, read, or understand English. C) Enrollment and data collection: Trained data collectors will screen older adults for eligibility, explain the project goals and rationale, answer questions, and obtain informed consent in person. After providing informed consent, participants will complete the UCLA Loneliness Scale and Demographic survey. Each participant will complete two 60-80-minute interviews with a member of the research team in a safe, private, and comfortable location of their choosing. During the first interview, the participant will complete a narrative interview. This interview will be unstructured, with initial conversation initiated by the researcher stating, “Tell me the story of your life, including your experiences with loneliness”. Prompts will be used during the interview to elicit more of the story. During the second interview, they will work with the research team member to complete the social network and environmental surveys. All interviews will be audio-recorded to ensure high-quality data collection. In addition, demographic information will be collected for each participant. D) Data analysis: Upon completion of the first interviews, audiotapes will be professionally transcribed. Each transcript will be verified by the researcher to ensure accuracy and de-identification to protect the privacy of participants and affiliations. Verified transcripts will be distributed to members of the research team and narrative analysis will be completed. During analysis, each researcher will read the transcript and identify significant portions of the text that represent important aspects of the participants’ life story and their relationship with loneliness. Members of the research team will then come together to discuss the analyzed transcripts and further identify and interpret significant patterns of meaning for each participant. Data obtained during the second interview will be analyzed in order to determine a social network and environmental interaction profile for each participant. Scores on the UCLA Loneliness Scale and demographic data will be used to contextualize findings from Aim 1.

Expected Outcomes: Analysis of the life stories and information gathered through the social network and environmental surveys of each participant can ultimately provide potential targets for personalized interventions aimed at reducing loneliness. Moreover, the approach we present here is essential for characterizing the multidimensionality of loneliness as a subjective state.

Potential Challenges and Alternative Strategies: (1) An important consideration when working with older adults is the duration of loneliness, which may be temporary or chronic [46]. One challenge for our studies is to ensure we are capturing all salient characteristics of loneliness, including duration. (2) A related consideration is the early identification of at-risk individuals. As with any disease or disorder, early assessment and identification is crucial for an effective intervention. (3) We anticipate that some participants may need to exit

the interview prior to completion. For instance, if a participant displays or states that they are emotionally upset, the interviewer will end the interview and refer the participant to a Maple Knoll employee who will be able to provide additional support. If the participant appears to become tired, the interviewer will check in to see if they want to continue or stop at that point. Consistent with our IRB protocol, no participant will be coerced to complete the interview if they become emotionally upset or tired. Each participant will receive a gift card incentive, even if they are unable to complete the interview. An alternative, should we encounter this situation, would be a reduction in the length of each interview and scheduling of additional shorter interviews. (4) Some components of loneliness may not be captured in the established measure. For instance, the 42 years since the development of the UCLA measure have demonstrated countless societal changes, including an increase in geographic separation of family networks and the rise of the internet and social media [47]. For this reason, narrative interviews are even more crucial as data-collection instruments.

Benchmarks for Success: Based on evidence from prior qualitative analyses conducted by our team members, individuals ascribe meaning to their currently felt loneliness by placing it in the context of their life-stories, as articulated through conversation. Therefore, the process of discussing their life stories, including previous and current experiences with loneliness, is anticipated to be revealing of loneliness's complex etiology and rewarding for participants. Once recruited, we anticipate that individuals reporting feeling lonely will provide information consistent with the principal measure: the UCLA Loneliness Scale. Although this measure is expected to provide incomplete insight into the experience of loneliness for these participants, consistent reports using this measure will confirm that our approach is valid.

Aim 2. Examine biological and physiological pathways associated with loneliness through comprehensive of gut microbiome analyses. The personal and unique qualities of loneliness can only be fully understood through comprehensive analyses inclusive of underlying biological factors. Biological contributions to loneliness have been understudied in the literature, perhaps owing to the challenge of teasing apart the unique effects of social relationships on cognition, mood, and physical symptoms. Here, we examine putative biological correlates of loneliness through comprehensive analyses of microbiome compositions in lonely and non-lonely persons. At present, the biological underpinnings of loneliness remain largely unknown. To inform this gap in the literature, we will evaluate the gut microbiome composition and abundance (see overview, Fig. 2) in a set of older adults self-reporting as lonely, and a set reporting as non-lonely.

A wealth of research in the past decade has led to an appreciation for the microbiome-gut-brain axis: an interconnected relationship between the commensal microbes in the gut and specific psychiatric conditions [17]. The consensus belief is that a 'healthy gut microbiota' core is comprised of *Firmicutes, Bacteroidetes, Actinobacteria,* and *Verrucomicrobia* [48]. The human microbiome is diverse, comprising microbes and metabolites that can be healthy or harmful. For example, persons suffering from IBS have imbalances in their microbiota; e.g., decreased abundance of *Bacteroidetes*, and increased abundance of *Firmicutes* [49]. Patients suffering from major depressive episodes demonstrate reduced abundance of *Firmicutes*, and increased abundance of *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* [50]. The question of precisely how the microbiome impacts and interacts with brain health remains unclear. At least four pathways, however, appear to be implicated, including: 1) regulation of immune activity; 2) alteration of tryptophan metabolism; 3) production of short chain fatty acids (SCFAs); and 4) direct production of neurotransmitters by gut microbiota

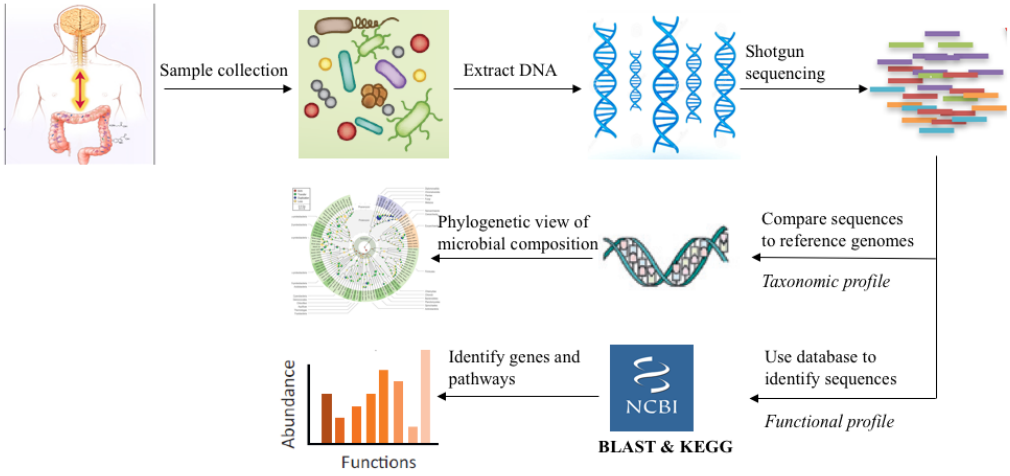

Figure 2. Summary of microbiome analyses of stool samples

[17]. A promising area of research is the potential role of SCFAs since they have been shown to cross the blood-brain barrier and have neuroactive properties [51].

Experimental Methods

2.1 Whole metagenome sequencing and taxonomic/functional profiling in lonely and non-lonely individuals. We will complete whole metagenome sequencing workflow for the 60 participants enrolled in our study. Participants will be classified as "lonely" or "non-lonely" based on self-report and previous administration of the UCLA Loneliness Scale. A) *Sample collection*: Once consented, each individual will self-collect stool and deposit it into a commode specimen collection system (Fisher Scientific). All stool samples will be placed in a styrofoam box and packed on ice. Collections will be communicated to the investigators and delivered to the laboratory via courier within 24 hours. In addition, each participant will work with a study team member in order to complete a brief survey to report their recent diet, medications, and a health history, including any diseases or other health issues that might influence the metabolic profile. Under aseptic conditions, all samples will be homogenized with a microspatula and separated into two roughly equivalent aliquots. Samples will then be flash frozen on dry ice and stored at -80°C until extraction. B) *DNA extraction*: extraction of nucleic acid from samples will be performed using 0.10 – 0.25 g of stool mixed with bacterial lysis buffer (including lysozyme and proteinase) using the Fecal DNA Isolation kit (MO Bio) for 10 minutes, and microbial DNA will be isolated by bead-beating for three minutes and purified using a Qiagen DNA spin column. Purified extracts will be stored in TE buffer, and concentrations of all samples will be determined using a NanoDrop spectrophotometer and stored at -20°C until further processing. C) *Shotgun metagenome sequencing*: To prepare a library, ~1-5 µg of genomic DNA will be mechanically sheared and size-selected to ~300-600bp fragments (Covaris LE220 or S220 instrument). Shearing conditions will be carried out using Crimp-Cap microTubes with AFA fiber, according to the following conditions: temperature: 7-9°C; duty cycle: 20%; intensity: 5; cycles per burst: 200; time: 90 s. Fragmented DNA will be end-repaired and 3'-adenylated and ligated using Illumina proprietary adaptor sequences. Libraries will then be subjected to whole-genome sequencing using the Illumina NextSeq 550 sequencing system (GES Core) or the Illumina HiSeq platform (CCHMC Sequencing Core). D) *Whole metagenome sequence analysis (taxonomic/functional profiling)*: We will use MetaPhlAn2 software [52] to perform taxonomic profiling to determine the composition of gut microbial communities (Bacteria, Archaea, Eukaryotes and Viruses) based on shotgun metagenomics sequencing data. This tool maps raw sequence reads to a database of predefined clade-specific marker genes and returns the taxonomic identity of each read cluster. After mapping clade-specificity, raw counts will be normalized to provide profiles of clade relative abundance and marker gene presence/absence. We will then use HUMAnN2 software (http://huttenhower.sph.harvard.edu/humann) to perform functional profiling of genes based on the KEGG database of gene families and pathways [52]. E) *Statistical Analysis*: We will use principal component/coordinates analysis (SPSS software) to reveal the clustering and correlation patterns between loneliness scales and relative abundance of each taxonomic rank. A p value <0.05 will be considered statistically significant.

2.2 Microbiota metabolite analyses based on short-chain fatty acid (SCFA) composition and abundance: Although there are a number of pathways that may mediate the interaction between the microbiome and the brain, we are pursuing a putative role for composition of SCFAs. Our rationale for exploring SCFAs is that aging is accompanied by a number of normative neurobiological changes. These changes include altered hypothalamic-pituitary-adrenal (HPA) axis function, and reduced circulating levels of neurotransmitters (i.e., two alternative pathways in the microbiome-gut-brain axis) [53]. It would be extremely challenging, therefore, to disentangle the effects of 'loneliness' versus 'aging' if we discover alterations in these two pathways. Moreover, we believe there is a plausible link between SCFA concentrations and brain functioning. For instance, injection of high doses of propionic acid to the central nervous system leads to a range of behavioral and neurodevelopmental alterations in rats [54]. SCFAs have also been shown to regulate the immune system in the gastrointestinal tract which may impact neurological functioning [55], as well as rescue microglial functioning [56]. Given this prior research, we believe that examination of the metabolite abundance is the most productive first approach. A) SCFA analysis: A panel of SCFAs and butyrate will be analyzed using gas chromatography-mass spectrometry (GC-MS), an analytical method to identify the constituent components of a test sample. Each test sample will be prepared from 1g of stool suspended in 2 ml of deionized water, and homogenized. This suspension will be centrifuged for 10 minutes at 10,000 x g. To the supernatant, we will add an internal standard, 4-methylvaleric acid solution. The supernatant will be analyzed through GC-MS or LC-MS. B) Data analysis: We will use SPSS and Origin 9.0 software to correlate SCFA concentrations with

loneliness scales and microbiota relative abundance. Both linear and non-linear correlations will be calculated using Pearson's and Spearman's coefficients.

Expected Outcomes: These experiments are anticipated to provide a profile of microbiome differences (specifically, in phyletic abundance and diversity) in our sample of participants experiencing loneliness, by comparison to those not reporting loneliness. We anticipate that this approach (see Fig. 2) may identify a link between the subjective experience of loneliness and alterations to the human microbiome.

Potential Challenges and Alternative Strategies: 1) The emerging concept of a microbiota-gut-brain axis is hypothesized to signal through four pathways. We believe that the metabolite pathway (comprising SCFAs) is the most likely pathway implicated in loneliness since they have neuroactive properties and are capable of crossing the blood-brain barrier. However, our results may not support this notion. Therefore, if we encounter discrepant results with our metabolic analyses, we will turn our attention to each of three alternative pathways, including: a) direct regulation of immune activity (and indirect production of cytokines) [57], b) alteration of tryptophan metabolism [58], and c) direct microbial production of neurotransmitters (e.g., GABA, dopamine, noradrenaline, acetylcholine) [59]. 2) The results we obtain may be highly complex, and subject to a number of confounding variables. For instance, loneliness co-occurs with depression [60], a disorder with known associations with gut microbiome diversity [61]. Therefore, we will need to carefully evaluate all study participants alongside a panel of relevant cross-sectional information to understand the potential influence of the many (potentially confounding) variables (medications, diet, mental and physical health conditions) known to impact the microbiome. A longitudinal design with structural equation can be used to distinguish the effects of loneliness from depression [62].

Benchmarks for Success: Since abundance is more often altered than composition, we anticipate that our first round of studies will capture the phyletic diversity of human microbiomes. Specifically, identification of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Verrucomicrobia* in our non-lonely cohort will confirm that our approach is valid, and we will proceed with data collection and analysis for the lonely cohort. Additionally, although our second Aim is not reliant on our first Aim, comprehensive in-depth interviews from Aim 1 will assist in interpretation of microbiome data, particularly with respect to the severity of discomfort for each individual.

Future Research Directions

The long-term goal of this research program is to develop a personalized, effective approach to treating and reducing the personal and societal impact of loneliness. As a step towards this goal, we seek to address each of three key challenges to addressing social isolation and loneliness in older adults. Jopling (2015) articulated these challenges as: 1) reaching or accessing individuals; 2) assessing the unique nature of a person's loneliness or isolation; and 3) facilitating access to services [63]. We anticipate that this research program, in partnership with our collaborators, will enable assessment of the psychosocial and environmental components of loneliness through a comprehensive, qualitative approach (Aim 1). Once completed, we will develop "profiles" of loneliness that capture the most critical psychological and social components of each individual. We then hope to develop a means of connecting established, evidence-based approaches and treatments to quickly and effectively mitigate loneliness. In the future, robust biomarkers of loneliness may be utilized to identify individuals at risk, enable early intervention), and provide an objective indicator to complement subjective data collection (Aim 2). The foundational work proposed here represents a comprehensive, transdisciplinary approach to tackling an important societal problem and is anticipated to provide us with the substrate for understanding unique loneliness at the individual level and developing an effective treatment paradigm for the broader population.