Acute Bioavailability of Berry Flavonoids and Impact on Inflammatory Biomarkers in Older Adults with Minor Depressive Symptoms
ABSORB

Study Protocol
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CHAPTER 1. INTRODUCTION

Blueberries are well recognized as a "superfood" due to their rich source of anti-oxidants and dietary fiber, and are recommended to consume as a part of a healthy diet. Regular consumption of blueberries as a source of dietary antioxidants may be an effective way to lower inflammation in older adults, who commonly have higher levels of inflammatory markers. However, older adults typically have a decreased efficiency of nutrient absorption and may need a higher dose of blueberries to absorb enough of the flavonoids needed to reap their benefits on inflammation. Thus, it is important for preliminary studies to pre-determine an appropriate dose of blueberry flavonoids specifically for older adults. This study aims to conduct a randomized crossover trial in 5 older adults. We will compare the bioavailability of flavonoids of a standard dose of blueberries (~1 cup) to a high dose (~2 cups) in older adults to help inform the design of future, larger studies in older adults.

CHAPTER 2. BACKGROUND

Inflammation: an Aging Risk Factor – Inflammation is now recognized as a key contributor to a plethora of age-related diseases and conditions that span across the human body,[1, 2] including cardiovascular disease, cognitive impairment, arthritis, etc. Older adults are particularly vulnerable to the effects of inflammation given they are often diagnosed with inflammatory-related diseases. Furthermore, inflammation itself typically increases in aging, a phenomenon commonly referred to as "inflammaging."[3] Given that inflammation is a key contributor to most age-related diseases and is a common phenomenon of aging, ***therapeutic strategies that aim to reduce inflammation as a means to alleviate the burden of age-related diseases in older adults are needed.***

Dietary Flavonoids to Target Inflammation – Conveniently, an individual's inflammatory status is highly modifiable by one's diet.[4] Several nutrients and dietary compounds have potent anti-inflammatory properties, which may help reduce inflammation. Flavonoids are anti-inflammatory compounds found in fruits and vegetables.[5] A large body of research indicates that flavonoids have anti-inflammatory properties and block the transcription factor, nuclear factor kappa B, which is the master regulator for the production of several inflammatory cytokines as reviewed by Ferraz et al.[6] Blueberries are a convenient, rich source of anti-inflammatory flavonoids. Research from several pre-clinical and a few clinical studies has demonstrated that blueberry flavonoids reduce reactive oxidant species and dampen inflammatory pathways.[5, 7-21] Thus, blueberry flavonoids hold great promise to reduce inflammation, as a therapeutic strategy to delay, prevent, or reverse age-related diseases and conditions in older adults.

The Gaps – Despite their promising potential, the dose of blueberry flavonoids needed to target inflammation in older adults is unknown. Studies from younger individuals estimate that between 1-2% of flavonoids from a standard dose of blueberries (~1 cup) are absorbed into circulation (i.e. bioavailable).[22-24] However, older adults have a decreased efficiency of nutrient absorption,[25, 26] and may need a higher dose of blueberries to absorb enough of the flavonoids needed to reap their benefits on inflammation. Comparing the bioavailability of flavonoids of a standard dose of blueberries (~1 cup) to a high dose (~2 cups) in older adults will be the first step to identifying an appropriate dose needed for older adults in future studies. A second crucial step to identifying the appropriate dose of blueberries, will be to determine if the flavonoids absorbed from the blueberry doses will be in sufficient quantities to modulate inflammatory processes. **Together, the data from this study will provide crucial information on the dose of blueberries needed for older adults to absorb enough flavonoids to target inflammation.**

CHAPTER 3: RESEARCH DESIGN

3.1 Study Objectives and Aims

Aim 1: Determine the acute bioavailability of two different doses of blueberry flavonoids in older adults. *We hypothesize that those who consume 48 g of blueberry powder will have a higher percentage of flavonoid*

metabolite biomarkers in urine after 3 days of freeze-dried blueberry powder consumption compared to those who consume 24 g.
Aim 2: Determine the acute impact of berry flavonoids on inflammation in older adults. *We hypothesize that after 3 days of freeze-dried blueberry intake, select inflammatory biomarkers (e.g., C-reactive protein, interleukin-6, interleukin-1 beta, tumor necrosis factor alpha) will be lower compared to baseline, and those consuming the 48 g of powder will have a greater reduction. Additionally, the reductions in inflammatory markers will correlate with increases in flavonoid metabolites.*

3.2 Overview

This will be an individual-level, unblinded, randomized, cross over pilot study in 5 older adults with minor depressive symptoms. Screened and eligible participants will turn in a 24-hour urine collection, perform baseline assessments, and provide a blood sample. Next they will be randomized to consume either the higher dose (48 g/day) of blueberry powder (which provides ~8 g/day of fiber and ~600 mg/day of anthocyanins) or the lower dose (24 g/day) of blueberry powder (which provides ~4 g/day of fiber and ~300 mg/day of anthocyanins) for 3 days (**See §5.1, Figure 1**). After consuming their randomly assigned powder for 3 days, participants will collect a 24-hour urine sample and come in to repeat baseline assessments and provide a blood sample at follow-up. After a two week washout period, participants will repeat the same assessments/procedures with the other dose of powder.

3.3 Inclusion and Exclusion Criteria

Our target population is older adults with minor depressive symptoms. Both older adults and individuals with depressive symptoms typically have higher levels of inflammatory cytokines, representing a population that could stand to greatly benefit from an effective anti-inflammatory dietary strategy. Individuals expressing an interest in participating after recruitment out-reach will be screened via telephone (e.g., at HRC or at participant's home). Participants will complete several assessments to confirm that they meet the following inclusion criteria.

Inclusion Criteria

- Men and women aged ≥65 years
- Depressive symptoms (defined as ≥4 and <16 points on the center for epidemiological studies depression-scale, CES-D) or the CES-D revised version (CESD-R)[29])

Exclusion Criteria*

Exclusion criteria have been selected to ensure safety and optimize compliance, while minimizing confounds due to overt disease or conditions that may significantly influence study outcomes. Exclusions are described below:

- Unwilling or unable to follow the study protocol
- Cognitive impairment (assessed via the telephone Montreal Cognitive Assessment) defined as individuals scoring <19 which suggests levels of mild cognitive impairment[30]
- Self-reporting a history of major depression, bipolar, schizophrenia, or other psychotic or neurologic disorders
- Self-reporting history of gastro-intestinal diseases/conditions e.g., of bowel resection, inflammatory bowel disease/syndrome, Celiac disease
- Self-reporting immune disorders, e.g., rheumatoid arthritis, cancer, and other immunocompromising conditions
- Self-reporting history of type 1 or type 2 diabetes
- Self-reporting any history of substance or alcohol use disorder
- Allergy to blueberries
- Self-reporting use of anti-inflammatory (e.g., fish oil or non-steroidal anti-inflammatory drugs) or immune-suppressant drugs
- Are excessive tea or coffee consumers (e.g., >3 cups/day)
- Recent and consistent use (within the last 3 months) of antibiotics
- Currently taking or advised during the intervention to take anti-depressants
- Current homicidal or suicidal ideation (assessed via the P4 Suicidality Screener[31])

*All deviations must be approved by the study sponsor

3.4 Number of Subjects and Study Duration

We aim to recruit a total of 5 individuals (both men and women) through local newspaper and internet advertisements, physician referrals, our registry of research volunteers, Hebrew SeniorLife (HSL) senior housing sites, and patient registries (e.g., HRC or Beth Israel Deaconess Medical Center). Participants will remain in the study for approximately 5 weeks.

3.5 Study Endpoints

To adequately assess the utility of flavonoids to target inflammation in aging, dietary interventions that 1) asses the bioavailability of flavonoids in older adults and 2) evaluate the acute impact of flavonoids on inflammation are needed. Together the data from this study will determine an adequate dose of berries needed to reach a physiologically relevant level of flavonoid metabolites for my future studies. In addition to advancing the science, this proposal will deepen my knowledge of the relationship between flavonoids and inflammation. Thus, I will be well-trained and have the pilot data needed to conduct a future study on the effect of berry flavonoids on inflammation and age-related disease in older adults.

Primary Outcomes – The primary outcomes for this study will be level of flavonoid biomarkers and inflammatory biomarkers.

1. **Flavonoid Biomarkers:** The bioavailability of total flavonoid biomarkers will be evaluated by absolute change in total flavonoid urinary metabolites. Urine will be stored at -80 degrees C until analysis by a reputable lab (e.g., the Metabolomics and Analytical Chemistry Research Core at the University of Arkansas for Medical Sciences) by metabolomic methods as previously described.[32] Pre- and post-change in total flavonoid urinary metabolites for each arm of the intervention will be calculated for each participant.
2. **Inflammatory Biomarkers**: For each dose, pre- post- Change in serum markers relevant to inflammation will be measured by a reputable lab (e.g., the University of Connecticut Metabolic Phenotyping Lab). A panel of several serum inflammatory markers including interferon gamma, interleukins 1B, 6, 8, and 10, and tumor necrosis factor alpha) will be measured using a commercially available Luminex (R&D Systems, Minneapolis MN) bead-based immunoassay C-reactive protein (CRP) will be measured using a commercially available high-sensitivity enzyme-linked immunoassay (ELISA, R&D Systems, Minneapolis MN) and will be our primary marker to reflect inflammation.

Additional secondary outcomes (e.g., safety) will be evaluated.

3.6 Study Intervention Products

Given that 100% purified anthocyanins are not commercially available, we will utilize a dietary source of anthocyanins. Blueberries are one of the richest sources of dietary anthocyanins and also conveniently concentrated with fiber. We will use freeze-dried powdered blueberries as the intervention product. The powder is packaged in 24 g amounts. Therefore, participants will be asked to consume either one or 2 packets of powder each day depending on what arm of the intervention they are on. They will be asked to consume their entire dose in the morning. Participants will be asked to consume their dose mixed with water (i.e., ~ 6-8 ounces for every packet of powder). The powder will be provided by the U.S. Blueberry Council.. See **§**5.3 for further information on dietary intervention.

CHAPTER 4 RECRUITMENT AND DATA COLLECTION

4.1 Recruitment Overview

Participants will be recruited from the Boston area community, including senior housing facilities in urban/suburban areas and research recruitment repositories. We will utilize a multi-pronged approach to meet our recruitment goals:

- We will recruit from the research repository that resides at HRC
- We will connect with social workers in and outside the HRC
- We will perform medical record reviews to identify potentially eligible individuals at the Hebrew SeniorLife (HSL) geriatric medicine practices.
- We will advertise through direct mailings to all residents of HRC's seven supportive housing facilities (over 3,000 residents).
- We will give presentations at each Hebrew SeniorLife (HSL) facility.
- We will use the Harvard Catalyst (CTSA) Shared Health Research Information Network (SHRINE) to identify volunteers from Harvard-affiliated hospitals and clinics
- We will advertise our study within numerous local media outlets, on HRC's Hinda and Arthur Marcus Institute for Aging Research and other websites (*e.g.*, Craig's List), and at www.clinicaltrials.gov

4.2 Informed Consent

All interested individuals will be asked to provide verbal consent to complete an initial eligibility screen during a phone conversation with study personnel. Potentially eligible participants will then schedule an in-person visit. Potential participants may be sent by email or conventional post (per request, and according to their preference) a copy of the informed consent form for them to review at their own pace prior to the in-person screening. Written informed consent will be obtained by study personnel at the beginning of the in-person visit. If the participant signs the consent form and is willing to participate, they will continue with the assessments for the pre-intervention visit.

4.3 Participant Withdrawal

Any participant who expresses a desire to discontinue participation in the study will be withdrawn at their request immediately. All data collected prior to withdrawal will be maintained in the study data set.

Additionally, a subject may be withdrawn from the study prior to completing all of the study related procedures due to the following conditions:

- Subject safety issues
- Failure of subject to adhere to protocol requirements (including low compliance with the intervention)
- Disease progression
- Subject decision to withdraw from the study (withdrawal of consent)

Withdrawn subjects may not reenter the study unless there are extenuating circumstances (e.g. family emergency or required travel out of town) that interfere with the start of the study powder. In this case, they may be scheduled to start over again. If new medical conditions arise or are exacerbated during the study intervention, the withdrawal of a participant will be evaluated by the PI.

4.4 Methods to Protect Participant Privacy

The following are the planned procedures for effectively protecting against and minimizing loss of participant privacy:

1. Phone screening will be conducted in a private office space.
2. Study visits will be conducted in private rooms.
3. Each participant will be given a unique study identification number and data will not include any of the participant's PHI.
4. All participant-identifying information will be stored and managed on a secured database server. The information will be password protected.
5. Participant confidentiality will be maintained in accordance with Health Insurance Portability and Accountability Act (HIPAA) regulations.

6. Only the PI, study personnel, and laboratory personnel approved by the IRB and authorized to view PHI will have access to the information.
7. PHI will not be used during discussion, presentation or publication of any research data.
8. Files containing PHI data collected for recruitment and screening purposes will be kept in locked, secured filing cabinets accessible only to designated study personnel (research assistants and investigators).

4.5 Minimization of Bias

This study is an unblind intervention so staff and participants are aware of their assigned intervention arm. However, to minimize analyst bias, biomarkers will be de-identified and analyzed by technicians unfamiliar with the participants or study phase.

4.6 Maximizing Compliance and Minimizing Attrition

At the start of an individual's study participation, he/she will be given a schedule of their study visits. Visits will be scheduled at a time of day that the participant determines is most convenient for them, and will repeated at the same time for each visit. Transportation will be provided for each visit as needed, snacks will be available, and stipends will be provided for each study milestone. If necessary, reminder calls will be made to participants on approximately 2 days prior to study visits.

Participants will be tracked throughout their enrollment. Each study visit will be documented. Some study visits will be followed with a brief telephone check-in to ask the participant questions about medication compliance, adverse effects, and their experience during the most recent visit. All calls to the participant and their feedback will be carefully tracked. Notes that may facilitate compliance, such as "call before 10 am," etc., will be kept in participant files.

We will employ specific strategies to maximize participation and compliance:

- ***Positive Framing about Benefits:*** Information will be presented in terms of the possible gains rather than the avoidance of losses as this is a more effective motivational approach.
- ***Feedback and Recognition of Progress:*** Participants will be acknowledged throughout their participation with thank you notes, and will be recognized for their contributions to the study through regular brief flyers/newsletters such as "Partners in Progress – Mobility and Falls updates". We will remain in close contact with individuals throughout their participation with follow-up calls each month.
- ***Incentives and Rewards:*** Participants will receive snacks at each visit, cards for achieving milestones, such as birthdays, holidays, etc.; and certificates of completion.

CHAPTER 5. RESEARCH METHODS

5.1 Participant Visit Schedule

All study visits will take place at the Clinical Research Laboratory at HRC, Roslindale, MA, an HRC-affiliated housing site or if possible at a convenient location for the participant. The table below provides a high-level overview of the study visits.

Visit	Purpose	Procedures	Duration	Location
Telephone Pre-screen	Evaluate initial eligibility criteria of an individual	• Telephone pre-screen script and eligibility questions • Depressive symptom questionnaire • Telephone Montreal Cognitive Assessment	about 45 min	Over the telephone

Pre-Intervention Visit	Informed Consent and Instructions for diet records and urine sample collection	• Informed consent • Medical history questionnaire/medication use/questions about harming one-self • Begin 2 week washout period • On last day of washout, collect 24 hour urine and begin 1-day diet record	about 45 min	Hebrew Rehabilitation Center (HRC) or convenient private location for participant
Visit 1	1st Baseline/Start eating assigned powder	• Turn in 24 hour urine and 1-day diet record • Fasted blood draw • Questionnaires about lifestyle, mood/feelings, sleep and relevant symptoms • Height, weight, vital signs • Assignment to 1st powder • Begin 3-day diet record • On last day of 3-day powder intake, collect 24 hour urine	about 60 min	HRC
Visit 2	1st Follow-up Visit	• Turn in 24 hour urine and 3-day diet record • Fasted blood draw • Questionnaires about mood/feelings, sleep, relevant symptoms • Begin another 2 week washout period • Vital signs and weight • On last day of washout, collect 24 hour urine and begin 1-day diet record	about 60 min	HRC
Visit 3	2nd Baseline/Start eating other assigned powder	• Turn in 24 hour urine and 1-day diet record • Fasted blood draw • Questionnaires about mood/feelings, sleep, relevant symptoms • Vital signs and weight • Assignment to 2nd powder • Begin 3-day diet record • On last day of 3-day powder intake, collect 24 hour urine	about 60 min	HRC

Visit 4	2nd Follow-up Visit	• Turn in 24 hour urine and 3-day diet record • Fasted blood draw • Questionnaires about mood/feelings, sleep, relevant symptoms • Vital signs and weight	about 60 min	HRC

Participant eligibility will be determined during a telephone screening.

Telephone Pre-screen (~45 minutes): Volunteers will be asked about mood/behavior, depressive symptoms, medical conditions, as well as other exclusionary criteria.

Center for Epidemiological Studies Depression Scale (CES-D and CES-D revised): Depressive symptoms will be assessed by the original CES-D[28] and the CESD-R,[29] which are validated questionnaires of questions regarding feelings of depression, worthlessness, loneliness, energy level, and fear. The original CES-D questionnaire is a validated 20-item assessment. It was more recently revised to the CESD-R, which changes some of the items to ask about symptoms that are more consistent with the updated Diagnostic and Statistical Manual of Mental Disorder-5 criteria for major depression. We will implement both questionnaires since the original CES-D is most widely used in the literature and due to the high internal consistency (r=0.90) and a test-retest reliability of 0.51 of the CESD-R.[33] For the purpose of this study, we will primarily use the CESD-R score to identify our target population (e.g., individuals with scores as ≥4 and <16 points) who will be eligible to continue with the study.

Telephone Montreal Cognitive Assessment (tMoCA) will be administered by a trained research assistant to determine if the potential participant has normal cognitive status. Individuals will be excluded if tMoCA scores <19 points, which is indicative of cognitive impairment/dementia.[34] The assessor will be trained and certified in the conduct of this test.

If eligible and interested, the participant will be asked to review and sign the consent form. The process of informed consent can take place at the study clinic or at a private location convenient for the individual (e.g., the individual's home).

Pre-Intervention Visit (Informed Consent, approximately 45 minutes): In order to participate in this study, all interested and eligible participants will be required to provide informed consent. They will be given ample time to ask any questions about the study. A trained research staff will answer any questions and if the individual is interested in participating in the study, they will be offered to sign the informed consent form. When the staff is confident that the participant is completely familiar with the document and understands all the aspects of the informed consent form, it should be signed by the participant in the presence of the staff member, and should then be signed by the staff member. All consent forms will be double checked to make sure they are properly signed and dated. Copies of completed consent forms will be given to the participant

and the original signed document will be kept on file at the Hinda and Arthur Marcus Institute for Aging Research. As a part of the informed consent process, potential participants will be clearly informed that this intervention is not a treatment option for depressive symptoms or depression, but rather studying short-term a dietary strategy for health. If seeking a treatment, they will be directed to their primary care.

If the participant signs the consent form and is willing to participant, they will be asked about their medical history and the participant's potential suicidal ideation will be assessed. The participant will asked to avoid consumption of foods that are high in anthocyanins and/or dietary fiber (e.g., blueberries) for two weeks (i.e., washout period). On the last day of their two-week washout period (the day before their 1st baseline visit), participants will be asked to collect a 24 hour urine sample and complete a 1-day diet record.

Medical History: Additional measures to characterize the participants will include existing conditions, previous medical conditions, current medications etc.

Suicidality: Suicidal ideation will be evaluated via the P4 Suicidality Screener, which is a validated 4-item questionnaire.[31]

Diet Records: 1-day and 3-day diet records will be filled out by participants, documenting the amount and types of foods/beverages ate during the allotted time. Records will be reviewed by research staff for accuracy and completeness. Records will be entered into a dietary analysis program (e.g., Nutrition Data System for Research) to estimate dietary intake of nutrients.

Visit 1 (1st Baseline Visit, approximately 60 minutes)

Prior to their baseline visit, individuals will be asked to also fast 12 hours prior to their scheduled visit. They will come to our clinical research laboratory, have a blood draw (up to 20 mL of plasma and/or serum) and be asked questionnaires about their lifestyle (e.g., smoking habits), current social network, sleep habits, positive/negative affect, and relevant symptoms (e.g., gastrointestinal symptoms, pain etc.). For safety depressive symptoms (via CES-D/CESD-R and PHQ-9) and suicidal ideation will be assessed (via P4SS). Height, weight, and vitals will be measured. They will turn in their 1-day diet record and their 24-hour urine sample. After participants will be randomly assigned to either consume a 24 g dose (equivalent to ~ 1 cup) or a 48 g dose (equivalent to ~2 cups) of freeze-dried blueberry powder mixed in water. Participants will be asked to consume their assigned dose in the morning for the next 3 days, while recording diet records and compliance during those 3 days. Immediately after they consume the last dose of their intervention powder (e.g., Day 3), they will begin to collect a 24 hour urine sample for the day prior to their next study visit.

Lifestyle: Alcohol use, smoking status, etc. will be collected via self-report.

Social Network: Subjective perception of social support and connectedness will be evaluated using a validated questionnaire.

Sleep Quality and Quantity: Sleep will be evaluated by a self-report questionnaire and/or sleep log diary.

Height: Height will be measured at the baseline visit using a stadiometer.

Weight: Weight will be measured using a digital Health-o-meter scale.

Vital Signs (e.g., seated blood pressure) will be measured at the baseline visit. After 3-5 minutes of rest, seated blood pressure will be measured twice with an automated cuff.

Blood (up to 20 mL) will be collected by a trained phlebotomist using sterile procedures. Blood will be processed and stored at -80 degree C for future analyses. Batch analyses of relevant inflammatory markers (e.g., C-reactive protein, interleukins) will be measured by a reputable lab (e.g., the Metabolic Phenotyping Lab at the University of Connecticut).

Relevant Symptoms: Information on relevant symptoms including gastrointestinal distress, appetite, pain etc., will be collected by self-report.

Compliance: Compliance with our dietary intervention will be evaluated throughout the study. Participants will be asked to log consumption of their powder and keep all of their used powder packets. At the study visit, participants will return all unused and used powder packets to estimate number of intended doses that were consumed.

Mood Questionnaires: Given that our population of interest is more likely to develop more severe forms of depressive symptoms and experience suicidal ideation, both will be monitored at in-person visits. Depressive symptoms will be monitored by the Patient Health Questionnaire-9 (PHQ-9)[35] and the CES-D/CESD-R[28, 29]. These are validated questionnaires to evaluate severity of depressive symptoms and depression level. We will also assess the participant's attitude via validated self-report questionnaire, Positive and Negative Affect Scale (PANAS).

Visit 2 (1st Follow-Up Visit, approximately 60 minutes)

Participants will turn in their 3-day diet records, compliance logs, all packets, and their urine sample, as well as have a fasted blood draw (up to 20 mL of plasma and/or serum). They will also be asked to complete the same questionnaires that were administered at Visit 1 (except for lifestyle behaviors), as well as measure their vitals and weight. Participants will then enter a second washout period to avoid consuming flavonoid-rich foods for 2 weeks.

Just as they did before the 1st Baseline Visit, participants will be asked to collect a 24 hour urine sample and begin filling out their 1-day diet record as described before.

Visit 3 (2nd Baseline Visit, approximately 60 minutes)

This Visit will be exactly like Visit 1 (i.e., fasted blood draw, questionnaires, measurements of vitals etc. except for height measurement and evaluation of lifestyle behaviors). However participants will be asked to consume the other amount of powder for 3 days.

Visit 4 (2nd Follow-Up Visit, approximately 60 minutes)

This Visit will be exactly like Visit 2 and will be the last Visit of the study.

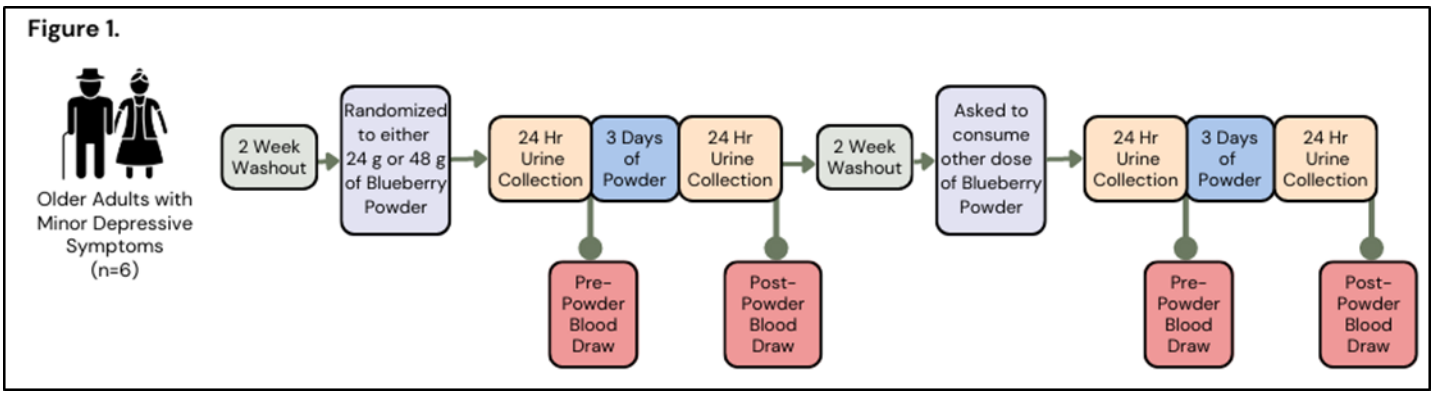
A summary of study visits and assessments is provided in the table below. Given that a primary goal of this pilot study is feasibility, we will allow for flexibility for the administration of some assessments at visits, as long as these changes do not impede the scientific interpretation.

Table 1. Assessments by Visit

	Telephone Pre-Screening	Pre-study Visit	Visit 1	Visit 2	Visit 3	Visit 4
Telephone Screening Script	X					
Depressive Symptoms[1]	X		X	X	X	X
Medical History	X	X				
P4 Suicidality Screener		X				
Informed Consent		X				
Diet Records		X	X	X	X	X
24-hr Urine Collection		X		X	X	X
Lifestyle			X			
Social Network and Sleep Habits			X	X	X	X
Height			X			
Vital Signs and Weight			X	X	X	X
Mood Assessment[2]			X	X	X	X

Symptoms	X	X	X	X
Blood Draw	X	X	X	X
Compliance		X		X

[1] via Center for Epidemiological Depression Scale (CES-D) and CES-D revised (CESD-R)
[2] Includes the Patient Health Questionnaire-9 and Positive and Negative Affect Scale



5.2 Study Visits and Assessments

This will be an acute bioavailability and biomarker study that consists of 4 study visits at our clinic (**Figure 1**). Screened, eligible, and interested participants (n=5) will be asked to avoid consumption of foods rich in flavonoids for 2 weeks (i.e., washout). Prior to their baseline visit, participants will be asked to collect a 24 hour urine sample to evaluate flavonoid metabolites and to fast 12 hours prior to their scheduled visit. Fasted participants (12-hour fast) will come to our clinical research laboratory, have a baseline blood draw and be randomized to either consume a 24 g dose (equivalent to ~ 1 cup) or a 48 g dose (equivalent to ~2 cups) of freeze-dried blueberry powder. Participants will be asked to consume their assigned dose in the morning for the next 3 days, while continuing to avoid other sources of flavonoids. The participants will be recommended to consume their assigned dose or mixed with 6-8 fluid ounces of water per packet of powder. After the last dose of powder is consumed on day 3, participants will begin collecting 24 hour urine and have a follow-up fasted blood draw the following day. After a second two week wash-out period, participants will be asked to repeat the same procedure with the other dose of blueberry powder so individuals can serve as their own control. During the study, participants will also be asked to complete diet logs to explore the impact of other foods/dietary habits on flavonoid bioavailability.

5.3 Dietary Intervention

During this intervention participants will be asked to consume a 24 g or 48 g dose of freeze-dried blueberry powder (in a randomized order), which will be provided by the United States Highbush Blueberry Council. A 24 g portion of blueberry powder is equivalent to approximately 1 cup of fresh blueberries and contains approximately 300 mg of flavonoids, and 48 g is equivalent to approximately 2 cups of fresh blueberries and contains approximately 600 mg of flavonoids. The powder is packaged in 24 g allotments. Thus, those consuming the 24 g will be asked to consume 1 packet of powder and those consuming 48 g will be asked to consume 2 packets. Therefore, the individuals and the researchers cannot be blinded to the allocation of intervention arm. Dietary flavonoids appear well-tolerated in older adults with no apparent adverse effects.[36] However, all adverse events will be appropriately documented.

5.4 Outcome Measures

The primary outcomes of this study are urinary flavonoid metabolite biomarkers (i.e., a marker of flavonoid bioavailability) and inflammatory biomarkers described below:

Flavonoid Biomarkers: Ingested flavonoids typically undergo extensive metabolism before and after absorption into circulation. Very little of the parent flavonoid structures (e.g., cyanidins) are absorbed intact, while the rest of the parent flavonoids are either extensively metabolized in the enterocyte or by

the gut microbiota into phenolic acids (e.g., hippuric acid, homovanillic acid, feurlic acid etc).[37-40] These phenolic acids are thought to be the active metabolites that are primarily responsible for the biologic effects of flavonoids.[41, 42] While these phenolic metabolites are in circulation, they are subject to further metabolism. Due to the extensive and complex metabolism that happens in circulation, most studies opt to use urine samples to evaluate bioavailability since select phenolic acids (primarily hippuric acid) appear to be the final metabolic end product of flavonoid metabolism.[43] It is estimated that approximately 50 major parent flavonoids and metabolites are detected in urine after ingestion of blueberries,[44] which have a moderate degree of inter- and intra-individual variability.[45] This high degree of variation is likely due to differences in lifestyle factors,[46] as well as biology/physiology between individuals. The bioavailability of total flavonoid biomarkers will be evaluated by absolute change in total flavonoid urinary metabolites. Participants will be asked to collect a 24 hour urine sample before and after powder consumption. Urine will be aliquotted and stored at -80 degrees C until analysis. Following the intervention period, frozen urine will be shipped overnight in bulk to a reputable lab (e.g., the Metabolomics and Analytical Chemistry Research Core at the University of Arkansas for Medical Sciences) where flavonoids and their flavonoid metabolic biomarkers are measured regularly. Urinary flavonoids and their metabolites will be measured by High-Performance Liquid Chromatography-Tandem Mass Spectrometry, as previously described.[47] The total flavonoid/metabolites pre- and post-intervention will be calculated for each participant.

Inflammatory Biomarkers*:* Given that it will take several hours for the flavonoids to reach the gut microbiota for metabolism and production of the active metabolites (i.e., phenolic acids), an acute 3-day feeding study was chosen to ensure enough time for 1) the metabolites to be formed via gut microbes and 2) a chance to modulate regulation of cytokine production. A panel of several serum inflammatory markers including interferon gamma, interleukins 1B, 6, 8, and 10, and tumor necrosis factor alpha) will be measured using a commercially available Luminex (R&D Systems, Minneapolis MN) bead-based immunoassay that allows for multiplex detection of several analytes in one sample. The Luminex will have ample sensitivity of the aforementioned markers range from 0.40-1.8 pg/mL, given that serum levels of the markers are typically >10 pg/mL.[48] C-reactive protein (CRP) will be measured using a commercially available high-sensitivity enzyme-linked immunoassay (ELISA, R&D Systems, Minneapolis MN) and will be our primary marker to reflect inflammation. The CRP ELISA has a sensitivity of 0.02 ng/mL which will be sufficient given that typically depressed individuals have levels >2 mg/mL.[48] The Luminex and ELISA will be performed by a reputable lab (e.g., our collaborators at the University of Connecticut). Pre-, post-intervention change in all measured inflammatory biomarkers will be calculated for each participant. All of the inflammatory biomarkers that will be measured have been previously linked with depressive symptoms,[48] and will thus be relevant to our recruited population.

CHAPTER 6. STATISTICAL DESIGN

6.1 Statistical Analysis

All analyses will be performed by intent to treat. Change in total urinary flavonoid metabolites and change in inflammatory markers will be calculated between baseline and the final follow-up (i.e., day 4 of each dosing cycle). The distribution of change in urinary flavonoid metabolites and serum inflammatory markers, will be summarized using sample quantities and kernel density estimates. Following this, formal inference and estimation of treatment effects will use a Student's paired t-test for comparison of urinary flavonoids and serum inflammatory markers between the 24 g dose and the 48 g dose. However, we will also explore other analytic methods that account for repeated measures. The correlation between changes in urinary flavonoid metabolites and changes in inflammatory markers will be assessed using scatterplot smoothing and summarized using regression analyses (linear or non-linear). Given the small sample size and pilot nature of the trial, we set a p-value threshold of 0.25 as suggestive of differences for all comparisons.[49]

We will also develop 80% confidence interval estimates of inter- and intra-individual variation (i.e., standard deviation) in these measures for sample size calculations for a subsequent clinical trial. The lower limit of the 80% CI of urinary flavonoid metabolites will provide a conservative estimate of the usual bioavailability of

flavonoids. In future studies, this lower limit will be used to estimate the amount/dose of berries needed to obtain a total urinary flavonoid metabolite concentration of ~1600 µg/mL, which coincided with the reductions in serum inflammatory marker, CRP in adults following a high-fat, high polyphenol meal challenge.

CHAPTER 7 DATA MANAGEMNT AND QUALITY

7.1 Data Management Plan

All data collected for analysis will be de-identified and assigned a unique study number. Any data collected on paper forms will be kept in a locked file cabinet at HRC. Data collected on paper forms will be entered and stored on a password-protected secure server at HRC. When possible, data will be collected directly via our electronic data capture system (e.g., REDCap).

The Institute for Aging Research primarily employs the REDCap system to facilitate data management operations. REDCap is a full-featured clinical trials data management system (DMS) accessible to data entry and data analysis workstations using secure Web technologies. The REDCap product is developed and maintained by Vanderbilt University in cooperation with REDCap Consortium members, including HRC. HSL hosts and maintains a dedicated instance of REDCap for use across our research enterprise. Each research study is provided separate project workspace in which all of the study data are stored in a MySQL relational database on the private corporate network behind several firewalls and located physically within the HSL data center.

7.2 Participant Tracking

Each recruited participant will be tracked closely throughout study enrollment. If desired, a study events calendar will be created for each participant. Any outstanding or incomplete visits will be accessible in real time to the project director and study team. The study team will maintain regular communications with each study participant throughout enrollment, through regularly scheduled follow up calls, and established retention strategies will be used.

CHAPTER 8 DATA SAFETY MONITORING PLAN

8.1 Participant Risks

Participation in this study may be associated with minor risks or safety concerns. The potential risks of this study fall into 4 categories: 1) those related to research participation; 2) those related to testing procedures; 3) those related to study powder; 4) those related to depressive symptoms. The risks are outlined for each category below:

Minor Risk of Participation in Research: With any study, risk of breach of confidentiality is possible since we are collecting personal health information.

Minor Risk of Testing Procedures: It is possible that participants may find the questionnaires tedious or may be uncomfortable being asked about sensitive topics like suicidality. The participants may also experience pain or bruising that results from the blood draw. With any puncture of the skin, there is an increased likelihood for infection, although this is minor. Participants may also find it inconvenient to fill out diet records and/or collect 24 hour urine samples.

Minor Risk Related to Study Powder: We do not anticipate any major risks for the participants with consumption of the freeze dried blueberry. However, the proposed intervention will provide an older adult with 30% of the recommended daily intake of dietary fiber. One may experience side effects that commonly occur with increased dietary fiber consumption if the individual typically consumes relatively low amounts of dietary fiber.

Risk Related to Depressive Symptoms: It is possible that individuals with depressive symptoms may progress in symptom severity during the study period. Although we do not anticipate a change to a more severe category of depression, we recognize that our proposed population of older adults with prevalent depressive symptoms are already predisposed to development of more severe depressive disorders. Importantly, our population of interest is also more likely to experience suicidal ideation.

8.2 Risk Minimization

We will attempt to minimize the identified risks as specified below:

Risk Minimization of Participation in Research: To minimize the risk of breach in confidentiality, all primary study data will be recorded with computer tablets on electronic case report forms (CRF) or as digital files generated from laboratory equipment. All data recording will be in accordance with procedures and guidelines outlined in the study's Manual of Procedures (MOP) authored by the study team. Participant confidentiality will be maintained by recording subject data using a unique subject identifier. Identifiable data, such as contact information and medical record numbers, will be recorded and stored separately from the clinical study data. Any paper-based study material and any identifiable data will be kept separate in a locked file cabinet accessible by authorized study staff only. Only the study staff directly responsible for the data collection and the safety of the participant will have access to identifiable information. All electronic CRF data will be stored securely in an electronic data capture and management system. Raw electronic instrumentation data will be organized and saved on a private network file dedicated to the research project. Only those listed on the approved IRB protocol will have access to subject data. Subject data will be coded and locked in a file cabinet in a locked office. Identifying information will not be used during discussion, presentation or research publication. All documents and electronic data will be archived for a minimum of three years, or as required by the IRB and federal regulations, after the completion of the clinical trial. The study will be registered at clinicaltrials.gov.

The Hinda and Arthur Marcus Institute for Aging Research employs the Research Electronic Data Capture (REDCap) system for data capture and data management operations. REDCap is a full-featured clinical trials data management system (DMS) accessible to data entry and data analysis workstations using secure Web technologies. While REDCap can be used to collect virtually any type of data (including 21 CFR Part 11, FISMA, and HIPAA-compliant environments), it is specifically geared to support online or offline data capture for research studies and operations. REDCap is developed and maintained by Vanderbilt University in cooperation with REDCap Consortium members, including HRC. HSL hosts and maintains a dedicated instance of REDCap for use across our research enterprise. Each research project is provided separate workspace in which all of the study data are stored in a MySQL relational database on the private corporate network behind several firewalls and located physically within the HSL data center.

Risk Minimization of Testing Procedures: The majority of the testing procedures will be questionnaire based. Participants will be advised that they can refuse to answer any of the questions. Participants will be permitted to rest between studies to prevent fatigue. To minimize the risk of being uncomfortable during questionnaires on sensitive topics like suicidality, only trained research staff will administer the questionnaires. Research staff will be trained to administer them in a calm, welcoming demeanor and will reassure the participants that the assessment(s) can stop at any time.

To avoid any risk associated with blood draws, only individuals with trained phlebotomist skills will draw blood using standard, sterile safety procedures to minimize risk of infection. Unless performed at the participant's home/residence or other HSL housing facility, blood draws will be done at our study clinic that resides in Hebrew SeniorLife, which is a functioning hospital. Thus, in the rare case a participant needs additional care that the study team is not qualified to provide, the individual will be transferred immediately to the adjacent hospital.

To avoid any inconvenience with collecting urine/filling out dietary records, all required equipment will be provided to the participant. If requested reminders for when to collect the urine sample/fill out diet records can be arranged.

Risk Minimization of Study Powder: Individuals with allergies to blueberries will not be included in this study to avoid any adverse effects/allergic reactions. We anticipate that the blueberry powder will be well-tolerated by participants since it consists of a food (i.e., blueberries) that are regularly consumed. Regardless, every visit we will ask participants about any complaints or adverse events that are directly related to the study intervention. We plan to track diarrhea, gas, bloating, abdominal pain, constipation etc. Since our intervention provides only ~30% of the recommended intake of daily fiber, we do not anticipate this amount will result gastrointestinal distress, if consumed alone. Nevertheless, symptoms of gastrointestinal distress will be captured at each visit and appropriately addressed by the study team. Dr. Millar, who has a PhD in Nutrition, will consult with participants on their experience with the fiber and reconcile any gastrointestinal stress. If a participant develops a health problem or a potential health problem (in addition to the ones outlined below), the PI will be notified ASAP.

Risk Minimization of Depression Severity: Although we do not anticipate a change to a more severe form of depression, we recognize that our proposed population of older adults with prevalent depressive symptoms are already predisposed to development of more severe depressive disorders. The level of depression will be evaluated and monitored at all visits to proactively monitor participant safety. If at any time during the study an individual's assessments indicate they have progressed to a more severe forms of depression, then specific safety protocols will be followed (e.g., notifying the PI). Our population of interest is also more likely to experience suicidal ideation, which will also be monitored at every visit to identify any individuals, who may need psychiatric care outside this study. If the participant is deemed to be dangerous or at imminent risk of harm, study staff will contact emergency services (i.e., #911) for immediate medical assistance.

General Risk Minimization: The proposed protocol requires 4 visits over approximately 5 weeks and therefore imposes a moderate amount of participant burden with respect to time and effort. Our institute has a strong track record of successful clinical research requiring similar participation, and retention has been high in these projects. The Clinical Research Laboratory at the Marcus Institute is located near a cafeteria and rest room, and is equipped with comfortable seating, a TV, movies, books, and magazines to keep individuals occupied during rest periods. Several additional strategies will be employed to minimize participant burden and maximize adherence to the protocol. We will:

- Develop a personal relationship between participants and members of the staff.
- Schedule appointments at convenient times with familiar staff.
- Explain to participants all aspects of their participation and follow up. We will demonstrate and practice study procedures before beginning data collection.
- Provide reminders of all appointments and follow-up phone calls.
- Include personal notes in the participant's data file to remember events in the life of the participant; these can be commented on at the next visit (e.g., birthday, birth of a grandchild).
- Provide snacks and lunch during all visits.
- Provide transportation for all visits, if required.
- Provide valet or dedicated on-site parking spaces.
- Compensate participants for visits.

8.3 Quality Assurance and Safety Monitoring

The Principal Investigator (PI) will assume primary responsible for ensuring participants' safety on a daily basis. Since this is a single-site, phase 1 pilot study, without high risk, our study will not require an official Data and Safety Monitoring Board (DSMB) or a Safety Officer. However, to ensure and monitor participant safety, any adverse event will be documented.

8.4 Adverse Event Collection and Reporting

Any adverse or serious adverse events will be logged using forms either provided by or modeled after the forms that are provided by the NIA Clinical Research Toolbox. The baseline visit will mark the beginning of when any potential adverse or serious adverse events could occur/be collected. All other AE's will be collected as a part of the parent study.

Adverse Event Definition and Categorization

An adverse event is any untoward medical occurrence in a participant, whether or not it is causally related to the study. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding, for example), symptom or disease temporally associated with the study. Adverse events will be recorded on the appropriate case report forms and source documents. The PI and/or trained staff member will evaluate all adverse events as to their severity and relation to the study. The severity of adverse events will be graded as follows:

Mild: Awareness of a sign or symptom but easily tolerated.
Moderate: Discomfort sufficient to cause interference with usual activity or to affect clinical status.
Severe: Incapacitating with inability to do usual activity or to significantly affect clinical status.
Life Threatening: The participant was at immediate risk of death from the adverse event as it occurred.

The Investigator will also assess the relationship of any adverse event to the study, based upon available information, using the following guidelines:

0 = Unlikely: No temporal association, or the cause of the event has been identified
1 = Possible: Temporal association, but other etiologies are likely to be the cause; however, involvement of the study procedures cannot be excluded.
2 = Probable: Temporal association, other etiologies are possible, but not likely.

To determine the attribution and temporal association of an adverse event we will consider the following:

1) Whether the participant reports they have experienced the same symptom prior to the study intervention.
2) Whether the symptom occurred and resolved within 24 hours of taking the study intervention.

The PI and study staff will consider the symptom according to the conditions stated above and determine temporality as per clinical judgment.

Definition of a Serious Adverse Event

A serious adverse event is any experience that results in any of the following outcomes:

- Death
- Is life-threatening
- Inpatient hospitalization or prolongation of hospitalization

A persistent or significant disability/incapacity. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse event when, based upon appropriate medical judgment, they may jeopardize the patient or participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. We do not anticipant any Serious Adverse Events with our intervention.

Adverse and Serious Adverse Event Reporting

There is a potential for adverse events and incidental findings during this study. A structured questionnaire asking about adverse events will be assessed during each visit of the intervention period. However, when any adverse has been identified, the study team will take appropriate action necessary to protect the study subject and then complete the Adverse Event form that will be modeled after the form provided by the NIA Clinical Research Toolbox. This form requires Principal Investigator review and signature. After review by the Principal Investigator any adverse event will be reported to the IRB as appropriate.

AE's that 1) are unexpected in nature, severity, or frequency, 2) are possibly, probably, or definitely related, and 3) suggests that the research places participants at a greater risk of harm than previously known or recognized, will be reported to the IRB as appropriate (e.g., within 2 weeks of the event).

If a serious event occurs, it will be brought immediately to the attention of the Principal Investigator, who will discuss the event with the co-investigators and together decide if immediate treatment is necessary. If such treatment is necessary, the PI, co-investigators, or the study staff will coordinate such treatment t an appropriate hospital or urgent care setting, contact the primary care physician, and notify the IRB. A Serious Adverse Event form that is modeled after the one provided by the NIA Clinical Research Toolbox will be completed, which requires Principal Investigator review and signature. If an AE is defined as a SAE, the Principal Investigator will be notified as soon as the event is known about. Routine reporting of any SAEs will be reported to the IRB within as appropriate.

Unanticipated problems or adverse events will be reported according to Advarra's IRB written guidelines for interventional studies. Unanticipated problems and serious adverse events that are probably, possibly, or definitely related to the study will be reported as soon as possible from the time of learning of the event, but reported within 10 days to Advarra's IRB per Advarra IRB guidelines. Advarra will be provided a written report submitted and a submission of the incident via the eIRB system. This form will record any adverse symptoms and/or study protocol deviations. Study staff will reference a Subject Safety Event Reporting Decision Chart provided and updated regularly by Advarra to determine whether an event needs to be reported to the Advarra IRB.

All other adverse events/study incidents will be logged on an Adverse Event log and reported to the IRB following the appropriate reporting times as defined by the Advarra IRB.

For less serious or incidental findings the Principal Investigator will speak with the participant about the finding. If necessary, the PI may suggest appropriate follow-up with their clinical provider and/or provide a letter describing the findings and need for follow-up.

Any adverse events that take place during testing will be reported to the PI and recorded in the database. The PI will have ultimate responsibility for monitoring participant safety in the trial. The investigators will be responsible for reviewing each adverse event in a timely fashion, and reporting all incidents to the appropriate regulatory agencies according to written guidelines.

8.5 Participant and Study Stopping Rules

Participant Stopping Rules: If a participant experiences any adverse event that is deemed "severe" as outlined in **§**8.4 (Adverse Events Collection and Reporting) their continuation in the study will be determined by the PI. Additionally, if a serious adverse event (SAE) occurs, it will be carefully reviewed by the study team. Any report of a serious adverse event (SAE) that is thought to be directly related to the study products or study procedures, will result in the participant's discontinuation from the study.

Study Stopping Rules: Similar to the participant stopping rules, all serious adverse events (SAE) will be carefully reviewed by the study team to determine if study termination is warranted.

8.6 Potential Benefits

Participants may not receive any significant health benefit from participation, although some may benefit from knowledge of their health status, as well as potential therapeutic effects freeze-dried blueberry powder.

8.7 Participant Compensation

Participants will be provided up to $200 stipend to compensate them for their time spent completing study procedures.

CHAPTER 9. TRAINING

A manual of operations will be created with standard participant instructions for each question and assessment. All research staff will review and sign the Site Signature Log – Delegation of Authority Log that is modeled after

the log provided by the NIA Clinical Research Toolbox to confirm their responsibilities related to the study. During startup, staff will undergo intensive training, and all training sessions will be logged and signed accordingly. They will conduct all study procedures on a few volunteers (more if necessary) with oversight from the PI to ensure consistency of raters and equipment setup. Quality checks will be done every six months throughout the data collection period.

Training will be based on standardized materials developed for the study, and coordinated by the Project Director/Study Coordinator. Every six months, the staff will undergo training review and quality checks on all assessments and drug distribution protocols. Additionally, any time there is an amendment to the study protocol, the change will be logged on a Change in Protocol Log. All study staff will be provided a summary of the protocol modifications and under-go re-training for the new protocol. The date, duration, and certification of all training will be documented and signed by the Principal Investigator on the appropriate training logs.

CHAPTER 10. REFERENCES

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