IMPACCT: "Investigating Monocyte Priming in Adults in a Caloric Challenge Trial"

Unique Protocol Identification Number: IRB00083476

National Clinical Trial (NCT) Identified Number: NCT05348395

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Summary of Changes from Previous Version:

Affected Section(s)	Summary of Revisions Made	Rationale
6.4 Study intervention compliance	Added verbiage to accommodate missed visits	Improve recruitment and retention by planning for missed visits
1.3 Schedule of Activities	Remove lipids, insulin, CMP blood draw at wk4 of experimental phase	Not needed
6.1 Study Intervention Administration	Revised run-in and experimental feeding requirements	Reduce barriers for participants by reducing the number of study visits
5 Study population	Revise BMI range for inclusion	Accommodate physically fit participants that may fall above the "normal" range BMI
1.3 Schedule of Activities (SOA)	Updated to include additional details of visit activities	Improve clarity of activities that will occur at visits
5.1 Inclusion	Added inclusion for waist circumference	Waist circumference will be helpful to determine eligibility, particularly for those that have an above "normal" range BMI
6.1 Study Intervention Administration	Reduced run-in feeding window	Reducing number of potential visits will improve efficiency for meal prepping and reduce participant burden

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STATEMENT OF COMPLIANCE

Provide a statement that the trial will be conducted in compliance with the protocol, International Council on Harmonisation Good Clinical Practice (ICH GCP) and applicable state, local and federal regulatory requirements. Each engaged institution must have a current Federal-Wide Assurance (FWA) issued by the Office for Human Research Protections (OHRP) and must provide this protocol and the associated informed consent documents and recruitment materials for review and approval by an appropriate Institutional Review Board (IRB) or Ethics Committee (EC) registered with OHRP. Any amendments to the protocol or consent materials must also be approved before implementation. Select one of the two statements below. If the study is an ***intramural*** *NIH study, use the second statement below:*

1. The trial will be carried out in accordance with International Council on Harmonisation Good Clinical Practice (ICH GCP) and the following:

    - United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, 21 CFR Part 312, and/or 21 CFR Part 812).

    National Institutes of Health (NIH)-funded investigators and clinical trial site staff who are responsible for the conduct, management, or oversight of NIH-funded clinical trials have completed Human Subjects Protection and ICH GCP Training.
    OR
2. The trial will be conducted in accordance with International Council on Harmonisation Good Clinical Practice (ICH GCP), applicable United States (US) Code of Federal Regulations (CFR), and the [specify NIH Institute or Center (IC) [Terms and Conditions of Award. The Principal Investigator will assure that no deviation from, or changes to the protocol will take place without prior agreement from the funding agency and documented approval from the Institutional Review Board (IRB), and the Investigational New Drug (IND) or Investigational Device Exemption (IDE) sponsor, if applicable, except where necessary to eliminate an immediate hazard(s) to the trial participants. All personnel involved in the conduct of this study have completed Human Subjects Protection and ICH GCP Training.
    For either option above, the following paragraph would be included:
    The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the IRB for review and approval. Approval of both the protocol and the consent form(s) must be obtained before any participant is consented. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. All changes to the consent form(s) will be IRB approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

1 PROTOCOL SUMMARY

1.1 SYNOPSIS

Title:	Mechanism of monocyte priming in humans- a feeding trial	
Study Description:		
Objectives:	Primary Objective:	Determine the molecular mechanisms by which high-calorie diets trigger monocyte priming and reprogramming in metabolically healthy human subjects and whether and to what extent these mechanisms differ from mice.
	Secondary Objectives:	To identify which proteins alter their S-glutathionylation status (and possibly their expression levels) in response to HCD-induced monocyte priming, and which signaling pathways are altered in these cells
Endpoints:	Primary Endpoint:	Monocyte priming will be assessed measuring MKP-1 activity Unbiased lipidomics
	Secondary Endpoints:	Monocyte protein *S*-glutathionylation state
Study Population:	Healthy adults, age 20-45	
Phase:		
Description of Sites/Facilities Enrolling Participants:	Wake Forest School of Medicine Clinical Research Unit	
Description of Study Intervention:	Feeding study with a run-in period followed by overfeeding on a Western diet	
Study Duration:	Up to 4 weeks of run-in + 8 weeks of experimental diet	
Participant Duration:	14 weeks	

1.2 SCHEMA

1.3 SCHEDULE OF ACTIVITIES (SOA)

Visit Week	T<-4	SV1 (<T -2)	SV2 (T -1)	Run-in week 1	RI-day 5	RI-2	RI-3	RI-4	EXP-1	EXP-2	EXP-3	EXP-4	EXP-5	EXP-6	EXP-7	EXP-8
Study activity																
Self-screen and/or Phone screen	x															
Consent		x														
Screen visit (SV) 1		x														
History and physical exam		x														
Screening labs (CMP, lipids, A1c, insulin)		x														
Past medical history		x														
Demographics		x														
Plate waste method orientation and homework		x														
Review study diet with RD		x	x													
Body weight		x	x													
Height		x														
Blood pressure		x	x													
Pulse		x	x													
Waist circumference		x														
Adverse events			x	x	x	x	x	x	x	x	x	x	x	x	x	x
Concomitant medications			x	x	x	x	x	x	x	x	x	x	x	x	x	x
Pregnancy test[5]		x	x													
Review plate waste homework			x		x	x	x	x	x	x	x	x	x	x	x	x
Calculate estimated energy needs[6]			x													
Start Run-in feeding				x												
Establish weight stability target					x											
Monocyte sample (blood draw)							x[3]		x[1]	x[1]	x[1]	X[1]	x[1]	x[1]	x[1]	X[1,4]
Body weight									x[2]	x[2]	x[2]	x[2]	x[2]	x[2]	x[2]	x[2]

Blood pressure									x[2]	x[2]	x[2]	x^2	x^2	x^2	x[2]	x^2
1- Samples are taken at the end of the week (i.e., 5-7 days of feeding)																	
2- Weight and blood pressure will be done once weekly in a fasted state																	
3- At least 5 days after establishing weight target, as long as the participant is ±2% of the weight target on the 5th day																	
4- Lipids, insulin, and CMP																	
5 – A urine pregnancy test will be conducted for all females of child-bearing potential																	
6 – Physical activity will be assessed in order to get Total Energy Expenditure (TEE) to estimate caloric needs																	

2 INTRODUCTION

2.1 STUDY RATIONALE

To determine the mechanism of monocyte priming in humans, we will do a complete feeding trial in 15 male and 15 female normal weight and metabolically healthy human subjects (20-45 years of age) using a western diet (WD), characterized as being high-saturated fat, high-fructose, and high-calorie for 8 weeks.

2.2 BACKGROUND

Monocytes and macrophages are essential for tissue homeostasis, but in the context of metabolic disorders they become dysfunctional and promote chronic inflammatory diseases, including atherosclerosis. However, the underlying mechanisms linking metabolism to altered phenotype remain poorly understood. We have shown that chronic exposure of blood monocytes to nutrient stress induced by a “Western”-style high-calorie diet (HCD) stimulates the formation of reactive oxygen species (ROS) and promotes protein thiol oxidation, resulting in monocyte dysfunction and the reprogramming of blood monocytes into a pro-inflammatory, pro-atherogenic phenotype, hyper-sensitive to chemoattractants. These metabolically “primed” blood monocytes give rise to re-programmed and dysfunctional macrophages, sensitive to oxysterol-induced cell death, with defective autophagy and dysregulated activation profiles. Monocyte priming by nutrient stress is mediated by the H2O2-dependent S-glutathionylation, inactivation and degradation of mitogen-activated protein kinase phosphatase 1 (MKP-1), a master regulator of both monocyte adhesion and migration and macrophage function and plasticity (Fig. 1). However, the source(s) of HCD-induced H2O2 and “oxidative stress” in “primed” blood monocytes has not been identified. Identifying this source of H2O2 may open new therapeutic avenues and the development of novel drugs for the prevention and treatment of cardiovascular diseases.

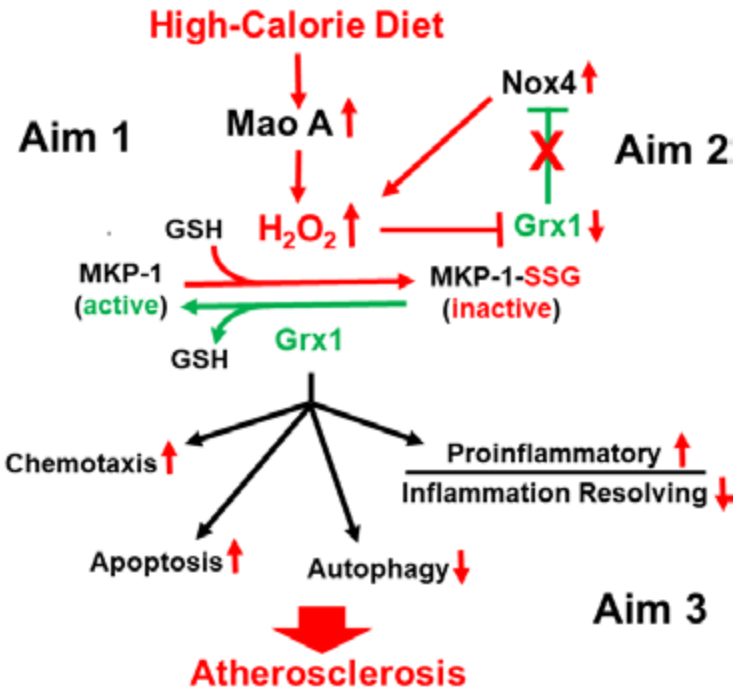

Figure 1: Central Hypothesis: Proposed roles for Mao A and Nox4 in high-calorie diet-induced monocyte priming and repro-gramming, macrophage dysfunction and atherogenesis. GSH: glutathione; MKP-1-SSG; S-glutathionylated MKP-1

Recently, we showed that a HCD also promotes monocyte priming, reprogramming and dysfunction in non-human primates. Primed baboon monocytes showed the same hyperchemotactic, proinflammatory and proatherogenic phenotype we identified both in HCD-fed mice and, ex vivo, for metabolically stressed human monocytes. In baboons, monocyte priming correlated with loss of MKP-1 activity in monocytes, validating MKP-1 activity as a sensitive biomarker of monocyte priming and dysfunction. More importantly, we identified a highly significant correlation between plasma cholesterol and both loss of MKP-1 activity and monocyte priming, suggesting that total plasma cholesterol may be the primary driver of monocyte priming, reprogramming and dysfunction. We will test this hypothesis in this Aim.

2.3 RISK/BENEFIT ASSESSMENT

2.3.1 KNOWN POTENTIAL RISKS

There is some minor discomfort and risk of mild bruising during venipuncture. A breech of participant confidentiality is an additional risk for each participant. Because this study involves overfeeding (i.e., providing more calories than necessary for maintenance of body weight), participants will be expected to see an increase in body weight. The expected weight gain is approximately 0.5 lb per week for an average person consuming 2000 kcal/day. We expect some participants will have adverse changes in blood lipids and markers of inflammation with the experimental diet and associated weight gain.

Discomfort associated with venipuncture is rapidly reversible. Bruises from venipuncture will heal in several days. To deal with the expected weight gain due to overfeeding, we will provide counseling at the completion of the trial to help participants lose weight back to baseline. We will provide this counseling free of charge through registered dietitians at our Weight Management Center. We will give participants guidance on a weight loss plan that includes a healthful dietary pattern that can reverse any metabolic changes seen during feeding.

2.3.2 KNOWN POTENTIAL BENEFITS

There are no direct benefits to individuals participating in this research trial.

2.3.3 ASSESSMENT OF POTENTIAL RISKS AND BENEFITS

Protection against risks: Risks to confidentiality will be protected by coding participant records as described above. Additionally, all study participant records will be kept in a locked file cabinet, in a locked office (in the Research Interviewer's office), and in a locked suite of offices that is protected by an automatic alarm system. When the Research Interviewer is not in her office, the office door will be kept locked.

All tests are conducted under the supervision of trained personnel. Since the tests are not inherently hazardous, hazard is likely to occur only as the result of impaired subject confidence or sudden unwillingness to complete the test. To avoid such hazards, study personnel thoroughly explain all tests to potential subjects before subjects are offered the consent/assent form to sign. Potential subjects have the opportunity to see all equipment and facilities before giving consent or undergoing testing. These precautions make it highly unlikely that harm will occur as a result of testing.

We will monitor the amount of weight gain each participant experiences on a weekly basis. Those individuals with much higher rates of weight gain than expected will have a review of their total energy expenditure estimates and study prescribed calorie goals. We will adjust the calories to stay within the approximate average range of weight gain for the course of the 8 weeks of feeding (i.e., stay within the goal of 25% higher calories than total energy expenditure). This should limit the potential for someone to gain excessive amounts of weight during the trial.

Because this trial includes an overfeeding component, and participants would be expected to gain weight during the 8 weeks of feeding, we will provide counseling at the completion of the trial to help participants lose weight back to baseline. We will provide this counseling free of charge through registered dietitians at our Weight Management Center. Participants will have access to counseling on a monthly basis for up to 12 months. If additional support is needed beyond this to assist with returning to a baseline weight, we will do an individual medical consultation with them and devise additional strategies to assist with returning to the baseline weight. Dr. Ard will also oversee this aspect of the trial.

Importance of knowledge to be gained: This study will help us understand how early changes in diet and calorie intake affect cells in the body that may ultimately initiate a series of changes that promote cardiometabolic disease risk. Diets that promote weight gain may act through a few key pathways to reprogram white blood cells and lead to abnormal activity within these cells. If we can identify these pathways, and more importantly, the triggers that initiate these events, we can potentially develop treatments that block the harmful effects of poor diets.

3 OBJECTIVES AND ENDPOINTS

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
Determine the molecular mechanisms by which high-calorie diets trigger monocyte priming and reprogramming in metabolically healthy human subjects and whether and to what extent these mechanisms differ from mice.	Monocyte priming will be assessed measuring MKP-1 activity Unbiased lipidomics	MKP-1 activity in blood monocytes is biomarker of metabolic stress-induced monocyte "priming" and dysfunction. We will analyze plasma from all subjects at all time points for their plasma lipid composition using an unbiased lipidomics approach in order to identify the lipid species responsible for monocyte priming
Secondary		
To identify which proteins alter their S-glutathionylation status (and possibly their expression levels) in response to HCD-induced monocyte priming, and which signaling pathways are altered in these cells	Monocyte protein *S*-glutathionylation state	In purified blood monocytes isolated from each subject at each timepoint . we will isolate *S*-glutathionylated proteins from all samples and subject them to our redox proteomics approach to identify S-glutathionylated proteins and top determine the extent and directionality of modifications on their cysteine residues.
Tertiary/Exploratory		
Determine the genes and pathways involved in monocyte priming in humans	RNAseq analysis	RNAseq will quantify mRNA expression of all transcripts in expressed monocytes at week 0, 1, 2, 3, 4 and 8, and any changes in their expression over time. These measurements will inform us on the proteins and pathways involved in monocyte "priming".

4 STUDY DESIGN

4.1 OVERALL DESIGN

The goal is to challenge metabolically healthy human subjects with a high-calorie, Western diet for 8 weeks to identify the metabolite(s) responsible for monocyte priming and use redox proteomics, RNAseq and Chipseq to determine the genes and pathways involved in monocyte priming in humans. To reflect some key components of the typical Western intake, we will compose the diet to be high in saturated fat (15% of total energy intake) and fructose (14% of total energy intake) with excess energy intake that is 25% higher than estimated total energy expenditure. A total of 30 participants, including 15 men and 15 women, will complete a run-in phase where weight stability will be achieved on the control diet. After weight is stabilized during the run-in period, participants will enter the experimental phase of the dietary intervention, where overfeeding will begin with additional calories provided by the key nutrients as noted above. Participants will be monitored throughout the feeding trial. After completion of the 8-week overfeeding period, individuals will be allowed to return to a normal dietary intake. All study participants will be given access to counseling for weight reduction should it be needed following the overfeeding period.

4.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

Recently, we showed that a HCD also promotes monocyte priming, reprogramming and dysfunction in non-human primates. Primed baboon monocytes showed the same hyperchemotactic, proinflammatory and proatherogenic phenotype we identified both in HCD-fed mice and, ex vivo, for metabolically stressed human monocytes. In baboons, monocyte priming correlated with loss of MKP-1 activity in monocytes, validating MKP-1 activity as a sensitive biomarker of monocyte priming and dysfunction. More importantly, we identified a highly significant correlation between plasma cholesterol and both loss of MKP-1 activity and monocyte priming, suggesting that total plasma cholesterol may be the primary driver of monocyte priming, reprogramming and dysfunction.

RNAseq analysis revealed that primed baboon monocytes undergo major reprogramming of their transcriptome and their signaling pathways (unpublished data). We also found major changes in the acetylation status of lysine residues histone 3 (H3). Acetylation of H3K27 strongly correlated with monocyte priming, suggesting that a four-week exposure to a HCD is sufficient to promote the transcriptional and epigenetic reprogramming of blood monocytes into a hyperinflammatory, proatherogenic phenotype.

5 STUDY POPULATION

5.1 INCLUSION CRITERIA

Age 20-45
Planning to be available for the entire study period
Able to speak and read English
Body mass index 18.5-29.9 kg/m^2
Waist circumference of 88 cm or less
Able to eat the prescribed diet
Non-smoker

5.2 EXCLUSION CRITERIA

Excessive alcohol consumption (>2 drinks/day for men, >1 drink/day for women)
History of chronic cardiometabolic disease or major risk factor, including diabetes, hypertension, hyperlipidemia, heart disease
History of prior surgical procedure for weight control or liposuction
Use of weight loss medications in previous 6 months
Recent self-reported weight change (≥ 5% in the last 6 months)
Severe pulmonary disease requiring supplemental oxygen
Abnormal renal or liver function
History of non-skin cancer in the past 5 years
Regular use of medications (prescribed or over-the-counter) that affect blood pressure, lipids, glucose, inflammation, or body weight.
Works night shifts
Exercise per week > 420 minutes total for moderate activity or > 210 minutes for vigorous activity
Any medical or behavioral indication that would make participation unsafe based on the judgement of the study physician
Pregnant or lactating women
Known or discovered intolerances, allergies or difficulty consuming any of the foods included in the study diets

5.3 LIFESTYLE CONSIDERATIONS

During this study, participants are asked to:

- Limit consumption of alcoholic beverages to recommended limits for men of no more than 2 drinks per day and for women no more than 1 drink per day

- Refrain from receiving outside treatment for obesity, including other treatment plans, medications, or pursuing surgery or other procedures
- Avoid pregnancy by reporting practice of one or more methods of effective contraception

Additionally, prior to certain study visits, participants will be asked to:

- Abstain from strenuous exercise for 12 hours before each blood collection
- Abstain from eating or drinking anything other than water for 8 hours before blood collection for clinical laboratory tests and study measurements.

5.4 SCREEN FAILURES

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently randomly assigned to the study intervention or entered in the study.

Individuals who do not meet the criteria for participation in this trial (screen failure) because of meeting one or more exclusion criteria that are likely to or do change over time may be rescreened. Examples include the successful treatment of a previous affective disorder, improvement of blood pressure control, and discontinuation of exclusionary medications. Rescreened participants will be assigned the same participant number as for the initial screening.

5.5 STRATEGIES FOR RECRUITMENT AND RETENTION

Recruitment

We will recruit 30 adult participants age 20-45, including 15 men and 15 women, for this feeding study. We will recruit the volunteers using several strategies proven successful in previous studies by the investigative team including general advertising in local media and the Wake Forest Be Involved website. Additionally, we will use local media outlets, including print, television, internet, and radio to generate interest in this research study, including those that target audiences of interest.

Retention

Participants will receive cash reimbursement of $150 for time lost from other activities to complete study related visits and measures. The money will be disbursed over the course of the study (3 x $50).

In addition to the monetary incentive we will provide to assist with retention, the research team will use strategies that have been effective in our previous clinical trials to promote retention. These strategies include recognizing individual milestones such as birthdays and providing personalized recognition of consistent levels of study participations. This type of public recognition encourages and reinforces the type of engagement we want to achieve for all volunteers. Our team will also follow up individually with

participants who miss appointments or meal times without prior notice to promptly reschedule or arrange for a make-up visit.

6 STUDY INTERVENTION

6.1 STUDY INTERVENTION(S) ADMINISTRATION

6.1.1 STUDY INTERVENTION DESCRIPTION

We will use a run-in feeding period of up to 14 days to establish a common baseline and ensure weight stability and adequate adherence prior to starting the experimental feeding component of the trial. We will provide participants with all meals and snacks during the study. The study diets will include a variety of foods and ingredients that are found in a typical American diet, including grains, meat, poultry, fish/seafood, fruit, vegetables, dairy, oils/fats, nuts, seeds, and added sugar. Because this menu and study protocol are not designed to accommodate special dietary needs, individuals with known food allergies, intolerances, or special dietary needs (e.g., religious accommodations) will be excluded from participation. Initial calorie intake will be determined based on estimated total energy expenditure using Mifflin St. Jeor with estimated activity factor. For female participants with a regular menstrual cycle, the start date will be based on last menstrual period (approximately 12-16 days since the end of the last period). The target weight for weight stability will be identified on the fifth day of the run-in diet. Once the participant is weight stable for 5 consecutive days, they will proceed to the experimental diet. Weight stability is defined as maintaining a weight within 2% of the weight achieved on the fifth day of the run-in diet. The run-in diet phase will be a minimum of 10 days (5 days to identify baseline weight + minimum of 5 consecutive days of weight within 2%). Those who have significant variations in weight (i.e., >2%) will have adjustment of calorie intake, and weight stabilization will restart. If weight stabilization is not in process by day 9 of the run-in diet, the participant will be dismissed. If participants demonstrate difficulty with study adherence during the run-in (e.g., unable to consume certain foods, missed appointments, incomplete reporting of food intake, etc), they will be excluded as well. The run-in diet will be a diet that is low in saturated fat (7% of calories) and fructose (4% of calories) and eucaloric to maintain current body weight.

After the run-in, participants will proceed to the experimental diet for 8 weeks of feeding. The experimental diet will provide 15% of calories from saturated fat and 14% of calories from fructose with a goal of providing 1.25x total energy needs. We will monitor adherence using direct observation, written daily food diaries, and food waste inventory (i.e., participants are trained to estimate portion of uneaten foods). Participants will be asked to pick up their meals from the Clinical Research Unit (CRU) 2-3 weekdays (e.g., Monday, Wednesday, and Friday), at which time they will receive meals and snacks for the remainder of that day and enough until the next scheduled visit.

6.2 DIETARY COMPOSITION

6.2.1 DIET FORMULATION- RUN IN

The run-in diet will provide adequate nutrition at various calorie levels designed to achieve weight maintenance for at least 5 consecutive days after the 5th day of feeding. The macronutrient balance of

the diet will include 45% of calories from carbohydrate, 35% from fat, and 20% from protein. This balance of macronutrients is consistent with a typical Western dietary pattern. Within the carbohydrate distribution of calories, 4% of the energy intake will be from fructose. Among the calories from fat, 7% will be provided by saturated fat. Unsaturated fat will be split between monounsaturated fat (14% of calories) and polyunsaturated fat (9%). An example of the macronutrient totals and calories provided based on sample calorie ranges for the run-in diet is shown below.

Total calories	**Kcal from total carbs (45%)**	**Kcal from fructose (4%)**	**Kcal from non-fructose carbs**	**Kcal from total fat (35%)**	**Kcal from saturated fat (7%)**	**Kcal from unsaturated fat**	**Kcal from total protein (20%)**
1200	540	48	492	420	84	336	240
1600	720	64	656	560	112	448	320
1800	810	72	738	630	126	504	360
2000	900	80	820	700	140	560	400

6.2.2 DIET FORMULATION- EXPERIMENTAL

The experimental diet will provide adequate nutrition at various calorie levels with an additional amount of total energy that is 25% higher than what was required to achieve maintenance of body weight during run in. The food groups and general food types used in the run-in diet will serve as the basis for the experimental diet. The additional calories will be added through increases in fructose and saturated fat primarily. In the experimental diet, fructose and saturated fat will account for 14% and 15% of total calories, respectively. Within the total carbohydrate and total fat calories, adjustments will be made in non-fructose and unsaturated fat calories to accommodate the additional fructose and saturated fat to allow for a distribution of macronutrients that is consistent with the run-in diet. An example of the calories from macronutrients and the associated changes in non-fructose and unsaturated fat calories is shown below.

1.25x calories	**Kcal from fructose (14%)**	**Kcal from saturated fat (15%)**	**Kcal from total carbs (45%)**	**Kcal from non-fructose carbs**	**Change in non-fructose carb kcals**	**Kcal from total fat (35%)**	**Kcal from unsaturated fat**	**Change in unsaturated fat kcals**	**Kcal from total protein (20%)**
1500	210	225	675	465	-27	525	300	-36	300
2000	280	300	900	620	-36	700	400	-48	400
2250	315	337.5	1012.5	697.5	-40.5	787.5	450	-54	450
2500	350	375	1125	775	-45	875	500	-60	500

Participants will gradually increase their consumption to achieve the 25%, so that they have time to adjust to the food volume and the amount of fructose in the experimental diet. It is expected that participants will be eating 1.25x their calories no later than 4 weeks into the experimental phase.

6.4 STUDY INTERVENTION COMPLIANCE

A daily food intake diary will be provided to participants that includes the menu for the day. Participants will be asked to notate anytime they consume one of the prepared food items or when they have something that is not part of the prepared menus. These diaries will be reviewed at each pickup. We will ask participants to estimate the portions of any uneaten food in the food diaries.
While the gold standard for estimating plate waste is manual weighing, this can be time-consuming and expensive. Standardized visualization techniques can take less time and have been used with great success in a range of studies. These visual estimation techniques have been tested for reliability and accuracy in many studies, producing results that closely approximate manual weighing methods.

Visual estimation techniques rely on separating food waste into different food components and having trained observers, in this case our study participants, estimate the proportion of food left over. One such scale, The Visual Comstock Scale, will be used to estimate food waste in this study. Using this method, food waste will be separated into its different food components and recorded in different values using a six-point scale ranging from 0%, 25%, 50%, 75%, 90% and 100%, where 0% represents none consumed and 100% represents all consumed. We may also use digital photography to record food waste, which can allow review of estimates of portion sizes by study team members, to ensure estimates are accurate and errors minimized.

If participants are having difficulty with any aspects of adherence, study staff will coach them to improve adherence. Those who are consistently unable to adhere to the feeding protocol will be dismissed from the study. Participants will be advised to maintain the same type of physical activity they did during the run-in period and avoid significant changes in overall activity patterns. Individuals with significant changes in physical activity levels compared to the run-in period will have adjustment of calorie intake to ensure that their calorie intake remains at 25% above total energy expenditure.

To corroborate the metabolic effects of the experimental diet on the study participants, we will complete lab work at week8, including a lipid panel, comprehensive metabolic panel, and insulin levels.

While 100% attendance of study visits is ideal, the study team realizes that situations arise where the participant may not be able to come in for a visit (i.e. illness or travel). If a participant is sick, traveling, or otherwise unable to attend in person visits, up to 1 week of missed visits will be allowed. However, missing visits will not be allowed in the first 3 weeks of the experimental phase since these are considered crucial weeks. If the participant is able to give advance notice to the study team, arrangements may be made with the CRU to accommodate continued feeding while they are away.

7 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

7.1 DISCONTINUATION OF STUDY INTERVENTION

The study intervention will be discontinued at the completion of the 8-week overfeeding period or at an early time point if the participant is unable to adhere to the feeding protocol.

7.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

Participants are free to withdraw from participation in the study at any time upon request. However, an investigator may discontinue or withdraw a participant from the study for the following reasons:

- If any clinical adverse event (AE), laboratory abnormality, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant
- Pregnancy
- If a participant has a break in feeding for more than 1 day in any week or more than 3 days over the course of the 8 week overfeeding period. A break in feeding is defined as consuming <50% of the provided food for the day.

The reason for participant discontinuation or withdrawal from the study will be recorded on the Study Withdrawal Case Report Form (CRF).

7.3 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if he or she fails to return for more than 1 scheduled visit and is unable to be contacted by the study site staff.

8 STUDY ASSESSMENTS AND PROCEDURES

8.1 EFFICACY ASSESSMENTS

Body weight will be monitored on a daily basis using a digital body weight scale provided to the participant at the beginning of the study. Participants will be asked to weigh at home in light clothing at approximately the same time each day.

Blood (20 ml) will be drawn from each subject once after the run-in period and once after each week on the experimental diet. Within 2 h after the blood draw, we will bead-isolate by positive selection (CD14+) blood monocytes using a RoboSep automated cell separator. We optimized this protocol for high yield (>95%) and high purity (>95%). Monocyte priming will be assessed measuring MKP-1 activity. To identify which proteins alter their S-glutathionylation status (and possibly their expression levels) in response to HCD-induced monocyte priming, and which signaling pathways are altered in these cells, we will isolate S-glutathionylated proteins from all samples and subject them to our redox proteomics approach. We will also measure Mao A and Grx1 protein expression by scWB analysis and Grx activity using [3H]BSA-SSG as a substrate to monitor for possible changes in Grx1 activity in response to the diet challenge. In addition, we will conduct RNAseq analysis to determine the genes and pathways involved in monocyte priming in humans.

8.2 SAFETY AND OTHER ASSESSMENTS

Our Data and Safety Monitoring Plan consists of several layers including ongoing monitoring by the Principal Investigator, Dr. Ard and staff who have contact with the participants, and the Institutional Review Board of WFU Health Sciences. First, we will hold bi-weekly meetings of all members of the clinical trial team where we will review participants' status. This meeting will be directed by Dr. Ard. Second, Dr. Ard and a designated sub-investigator will be providing medical oversight of the feeding study. He will oversee each participant's progress and medical history. If a participant is observed to be in threatening health (regardless of whether it is related to the study or not) or describes a serious adverse event, his or her physician will be notified. The participant will be discontinued from the study in this situation. Third, all unanticipated problems and serious adverse events will be reported to the Institutional Review Board at WFU Health Sciences per institutional policy. A plan for regular ascertainment of adverse events will include bi-monthly symptoms forms collected at planned study visits.

8.3 ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

8.3.1 DEFINITION OF ADVERSE EVENTS (AE)

An adverse event (AE) is defined as any untoward or unfavorable medical occurrence in a human subject, including any clinically significant abnormal sign (for example, abnormal physical exam or

laboratory finding), symptom, or disease, temporally associated with the subject's participation in the research, whether or not considered related to the subject's participation in the research.

8.3.2 DEFINITION OF SERIOUS ADVERSE EVENTS (SAE)

Consistent with NIH guidelines and OHRP policy, SAEs are adverse events that meet any of the following criteria:

- fatal or life-threatening,
- result in significant or persistent disability,
- require or prolong hospitalization,
- result in a congenital anomaly/birth defect, or
- are important medical events that investigators judge to represent significant hazards or harm to research participants and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition (e.g. hospitalization, death, persistent disability).

8.3.3 CLASSIFICATION OF AN ADVERSE EVENT

8.3.3.1 SEVERITY OF EVENT

Once reported, all AEs will first be assessed by the site clinician using a grading system, both in terms of severity and duration. Clinicians will categorize each AE as follows:

- Mild or moderate in severity based on its potential to impact the health and well-being of the participant. Details of what constitutes a mild vs. moderate severity AE will be outlined in the MOP but this will also be at the discretion of the site clinician. All SAEs will be deemed serious.
- Resolved or ongoing at the time of each clinical report.
- Brief (<1 day), Intermediate (1-29 days), or Chronic (≥30 days) based upon duration of reported AE.

8.3.3.2 RELATIONSHIP TO STUDY INTERVENTION

In addition to categorizing the severity of each AE, the study clinician will also indicate whether each AE was likely to be "unrelated" or "possibly related" to the study intervention (e.g. could it be related to the dietary intervention?). Relatedness determination will be made based upon several factors:

- The AE is an expected / known effect of the study interventions or a manifestation / symptom of an expected / known effect
- The presence of a temporal relationship between the study intervention and the AE
- No other known cause exists that is more likely to be responsible for the AE in question

Determination of relatedness should be supported by the data collected by the clinician. In general, the study clinician will err on the side of indicating relatedness unless there is a strong argument to the contrary (e.g. a sprained ankle sustained in a soccer game).

8.3.4 TIME PERIOD AND FREQUENCY FOR EVENT ASSESSMENT AND FOLLOW-UP

At each in-person study visit, including the final study visit, participants will be asked to complete a brief screener to ascertain for AEs, as well as potential SAEs.

8.3.5 REPORTING OF PREGNANCY

Pregnancy Incidentally Detected at Baseline Visit

Women found to be pregnant on baseline laboratory examination will not be permitted to enroll in the study.

Pregnancy after Randomization

Women found to be pregnant after randomization, or those who spontaneously report a new pregnancy during study follow-up will have this event documented in their participant record and their participation in the study terminated.

8.4 UNANTICIPATED PROBLEMS

8.4.1 DEFINITION OF UNANTICIPATED PROBLEMS (UP)

The Office for Human Research Protections (OHRP) considers unanticipated problems involving risks to participants or others to include, in general, any incident, experience, or outcome that meets **all** of the following criteria:

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the Institutional Review Board (IRB)-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied;
- Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

8.4.2 UNANTICIPATED PROBLEM REPORTING

To satisfy the requirement for prompt reporting, UPs will be reported using the following timeline:

- UPs that are serious adverse events (SAEs) will be reported to the IRB and to the study sponsor/funding agency within 10 days of the investigator becoming aware of the event

- Any other UP will be reported to the IRB and to the study sponsor/funding agency within 15 days of the investigator becoming aware of the problem

9 STATISTICAL CONSIDERATIONS

9.1 STATISTICAL ANALYSES

9.1.1 GENERAL APPROACH

For the RNAseq analysis, we are underpowered for differential analysis of individual RNA abundance values, but we anticipate 80% power to detect differences between integrated transcriptomic pathways at $\alpha = 0.005$ (10 pathways and a mean difference of 1.35 SD) with 15 subjects per group. To obtain a more complete picture of the extent of monocyte reprogramming induced by a HCD, we will integrate the redox proteomics data with the RNAseq data. We anticipate that the combination of these approaches will identify novel signaling pathways dysregulated by HCD and possibly new therapeutic targets to prevent monocyte dysfunction and reprogramming. As in our baboon study, we will analyze by scWB the acetylation state of H3 lysine residues (H3KnAc). If we identify a similar correlation between the acetylation of a specific H3 lysine residue, e.g. H3K27Ac, in these HCD-challenged human monocytes as we did in baboons, we will conduct Chipseq targeting specific H3KnAc to determine which genes identified by RNAseq are regulated by this epigenetic reprogramming. Finally, to identify the metabolite(s) responsible for monocytes priming, we will conduct unbiased metabolomic analyses on plasma of these subjects and validate any candidate metabolites for their ability to prime healthy human monocytes in our cell culture model of monocyte priming. Quantitative data will be analyzed with parametric and nonparametric two-sample and multi-sample tests, such as t-tests or Mann-Whitney, correlative analyses, analysis of variance or Kruskall-Wallis, or other linear models, depending on the distributional properties of the outcome measures and sample sizes. If the overall test is significant, we will select appropriate post-hoc analyses based on the comparisons to be made (all groups or interventions versus controls), sample sizes (unequal versus equal), and equal versus unequal variances across groups. The overall significance level will be 0.05.

10 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

10.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

10.1.1 INFORMED CONSENT PROCESS

Written informed consent will be obtained from all participants during the baseline study visit.

10.1.1.1 CONSENT PROCEDURES AND DOCUMENTATION

A trained staff member or investigator will give an overview of the study, describe the intervention components and data collection requirements, and encourage potential participants to ask questions. All potential participants will be informed of the study design and interventions, the risks and benefits of participation, their rights and responsibilities as research participants, and alternatives to participation. They will also be informed that participation is voluntary and that they can withdraw from the study if their initial or on-going experience makes it oppressive, burdensome, or otherwise uncomfortable. Potential participants will be asked to read the informed consent form and to ask questions. The form will be written in simple, easy-to-understand language. As a part of the consent process, study staff will be required to review all key aspects of the study verbally and will be provided with a structured checklist for this purpose. A copy of the signed and dated consent form will be given to participants, and the original document will be placed in the participants' individual study files, which will be stored in a secure location at the clinical site.

10.1.2 CONFIDENTIALITY AND PRIVACY

Confidentiality will be protected by collecting only information needed to assess study outcomes, minimizing to the fullest extent possible the collection of any information that could directly identify subjects, and maintaining all study information in a secure manner. To help ensure subject privacy and confidentiality, only a unique study identifier will appear on the data collection form. Any collected patient identifying information corresponding to the unique study identifier will be maintained on a linkage file, store separately from the data. The linkage file will be kept secure, with access limited to designated study personnel. Following data collection subject identifying information will be destroyed by deleting any data files three years after study closure, consistent with data validation and study design, producing an anonymous analytical data set. Data access will be limited to study staff. Data and records will be kept locked and secured, with any computer data password protected. No reference to any individual participant will appear in reports, presentations, or publications that may arise from the study.

10.1.3 FUTURE USE OF STORED SPECIMENS AND DATA

Participants will provide consent for storage of blood samples will have a sample of blood stored with a unique identifier that will not include any identifiable information. The unique identifier will be a randomly assigned number and only the principal investigator will have access to the code that links the unique identifier to the study participant.

10.1.4 KEY ROLES AND STUDY GOVERNANCE

Staff member	Role(s)
Principal Investigator	Study clinician, study oversight, informed consent
Study coordinator	Maintenance of regulatory documents; reporting to IRB; informed consent; recruitment and screening
Investigator team	Review of data, outcomes, and safety events
Research assistant	Data collection, data entry, informed consent, recruitment and screening
Study dietitian	Participant monitoring, dietary adherence

10.1.5 SAFETY OVERSIGHT

The principal investigator will be responsible for the overall monitoring of the data and safety of study participants. The principal investigator will be assisted by other members of the study staff.

10.1.6 CLINICAL MONITORING

Dr. Ard and a designated sub-investigator will be providing medical oversight of the feeding study. He will oversee each participant's progress and medical history. If a participant is observed to be in threatening health (regardless of whether it is related to the study or not) or describes a serious adverse event, his or her physician will be notified. The participant will be discontinued from the study in this situation.

10.3 ABBREVIATIONS

AE	Adverse Event
ANCOVA	Analysis of Covariance
CFR	Code of Federal Regulations
CLIA	Clinical Laboratory Improvement Amendments
CMP	Clinical Monitoring Plan
COC	Certificate of Confidentiality
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
DCC	Data Coordinating Center
DHHS	Department of Health and Human Services
DSMB	Data Safety Monitoring Board
DRE	Disease-Related Event
EC	Ethics Committee
eCRF	Electronic Case Report Forms

FDA	Food and Drug Administration
FDAAA	Food and Drug Administration Amendments Act of 2007
FFR	Federal Financial Report
GCP	Good Clinical Practice
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
GWAS	Genome-Wide Association Studies
HIPAA	Health Insurance Portability and Accountability Act
IB	Investigator's Brochure
ICH	International Conference on Harmonisation
ICMJE	International Committee of Medical Journal Editors
IDE	Investigational Device Exemption
IND	Investigational New Drug Application
IRB	Institutional Review Board
ISM	Independent Safety Monitor
ISO	International Organization for Standardization
ITT	Intention-To-Treat
LSMEANS	Least-squares Means
MedDRA	Medical Dictionary for Regulatory Activities
MOP	Manual of Procedures
MSDS	Material Safety Data Sheet
NCT	National Clinical Trial
NIH	National Institutes of Health
NIH IC	NIH Institute or Center
OHRP	Office for Human Research Protections
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SMC	Safety Monitoring Committee
SOA	Schedule of Activities
SOC	System Organ Class
SOP	Standard Operating Procedure
UP	Unanticipated Problem
US	United States

10.4 PROTOCOL AMENDMENT HISTORY

Version	Date	Description of Change	Brief Rationale
