CLINICAL RESEARCH PROJECT **Protocol #16-H-0129**
IND #: NA

Note: Supplement, Nicotinamide Riboside, is not subject to IND regulations as covered by the Dietary Supplement Health and Education Act of 1994.

NHLBI Protocol: ***Study to Evaluate the Effect of Nicotinamide Riboside on Immunity***

Short Title: Nicotinamide Riboside on Immunity

Keywords: Inflammasome, Sirt3, Nicotinamide riboside, Fasting,

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Subjects in study at NIH:	Number	Sex	Age range
	42	M/F	18-39 years

Multi-center trial:	No
Ionizing Radiation for Research:	No
Off-Site Project:	No
DSMB Involvement:	No
Tech Transfer:	CRADA
IND/IDE:	No

Table of Contents

1. Précis

Intermittent caloric restriction or fasting has numerous health effects including the reduction in numerous cardiovascular disease risk factors. The cellular programs activated by caloric restriction are similarly turned on in preclinical studies in response to a 24-hour fast. We have found that a beneficial effect of 24-hour fasting is that it blunts the activation of a component of the immune system, termed the Nod-like receptor family protein 3 (NLRP3) Inflammasome. This inflammasome, as a mediator of sterile inflammation, is associated with the development of diabetes and atherosclerosis. At the same time, we found that refeeding after the 24-hour fast significantly increased NLRP3 protein levels, IL-1β, and TNF signaling, and that fasting blunted the NLRP3 inflammasome response, in association with the activation of a fasting sensing protein called SIRT3.[1] Interestingly, a recently discovered naturally occurring form of vitamin B_3, called nicotinamide riboside (NR), has been found to activate SIRT3.[2, 3] We found that NR reproduces the NLRP3 inflammasome blunting effect of fasting when administered to primary human monocytes/macrophages in culture. Putting this together, it would be interesting to evaluate whether the administration of NR to human subjects would replicate the fasting blunting effect on the NLRP3 inflammasome. Interestingly, at the same time, it has recently been found, in a preclinical study, that the NLRP3 protein can orchestrate differentiation of naïve T- cells into Th2 cells. We therefore propose to more broadly examine the effects of NR administration on myeloid and lymphoid cell biology in healthy volunteers.

2. Background

Atherosclerosis and type 2 diabetes are associated with 'sterile inflammation' that exacerbates vascular injury and insulin resistance. A mechanism underpinning this involves activation of the innate immune system by cell surface receptor recognized damage-associated molecular patterns (DAMPs), which in turn, activate intracellular inflammatory proteins (the NLRP3 inflammasome). Inflammasome activation increases the production and release of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and IL18. This emerging link between the immune system and metabolic diseases raises the question as to whether caloric restriction activated pathways, which ameliorate consequences of metabolic disease,[3, 4] may directly suppress the NLRP3 inflammasome.

Emerging data finds that mitochondrial integrity and function control multiple immunological effects including effects on activation of the NLRP3 inflammasome in monocytes/macrophages, on T-cell polarity and in the activation of mast cells. At the same time data show that fasting and caloric restriction improve mitochondrial integrity, in part via activation of sirtuin proteins[4] and that caloric restriction blunts systemic inflammation.[5, 6] Conversely, overnutrition is linked to activation of the NLRP3 inflammasome (sterile inflammation) and T_H2 linked diseases including asthma. Putting this together, we reasoned that fasting, via maintenance of mitochondrial integrity, would dampen the NLRP3 inflammasome compared to the effects of refeeding. This hypothesis was explored in an initial NHLBI protocol (14-H-0103) where we found that the

NLRP3 inflammasome was lower in the fasted compared to the refed state and that Sirtuin 3 (Sirt3) activation played a role in the fasting effect. Refeeding itself also primed inflammatory pathways including the NLRP3 pathway and TNF signaling. Interestingly, serum obtained from the patients in the fasted and refed states recapitulated the effects of the inflammasome in human derived THP-1 monocytes and the administration of a Sirt3 activator (Nicotinamide Riboside - NR) could replicate the anti-inflammatory effect of fasting when administered to primary human monocytes/macrophages.[1] NR is a naturally occurring form of vitamin B_3, and it has been shown to be a Nicotinamide Adenine Dinucleotide (NAD^+) precursor that boosts cellular NAD^+ levels. NAD^+ acts as an activator and substrate for sirtuins.

From our prior study we concluded that: (i) fasting and refeeding differentially regulate NLRP3 inflammasome activation in humans; (ii) that the activation of Sirt3 by NR may have beneficial effects on inflammasome linked diseases and (iii) that fasting, refeeding and the activation of Sirt3 may play a broader role in the modulation of immune function.

To pursue the clinical potential of Sirt3 activation on immune cell modulation we propose to explore the administration of NR to healthy volunteers to explore the effects on myeloid and lymphoid cell biology.

Additional studies have been initiated or planned using NR to study effects on mitochondrial biology related conditions including aging (at the University of Colorado in Boulder – actively recruiting) and in heart failure (University of Washington in Seattle – protocol in developmental stage). Based on blood levels of NAD^+ from a pilot study performed at the University of Iowa, the dose of NR chosen by aging and heart failure studies is 1 gram daily. The PI (Sack) has also discussed the experience to date with Drs Christopher R. Martens (UC – Boulder) and Rong Tian (U. of Washington). As of May 17, 2016 the Boulder team has enrolled 20 subjects of whom 10 subjects had been taking 1 gram of NR daily with no evidence of intolerance or side effects. The Seattle team has completed a pharmacokinetics study (NCT02689882) on 8 subjects taking incremental doses up to 1 gram bid up to 9 days. This study revealed no significant side effects and enrollment of the first subject on the heart failure study had been initiated. We therefore propose to use the 1 gram daily dose.

3. Hypothesis

Nicotinamide riboside, as a Sirt3 activator, will mimic the beneficial immune effects of prolonged fasting and suppress immune activation of refeeding.

Proposed Scientific/Clinical Innovations and Advances of the Study

- Evaluate whether pharmacologic activation of Sirt3, via enhancing mitochondrial integrity will blunt sterile-inflammation linked NLRP3 inflammasome activation.
- Expand our understanding how NR can modulate the regulation of additional innate and adaptive immune pathways

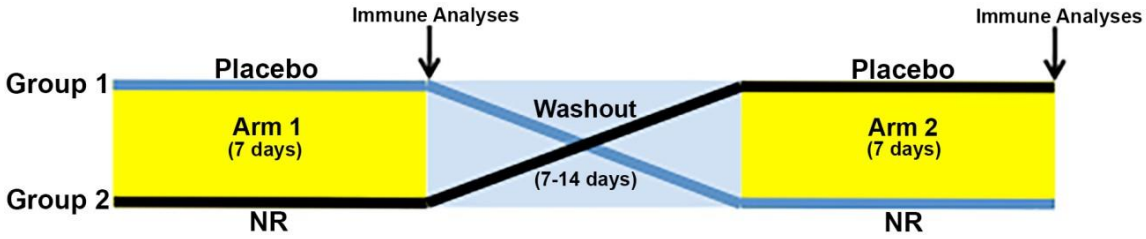
4. Objectives

1. Explore whether NR administration will blunt NLRP3 activation in human subjects.
2. Explore whether NR will modulate additional innate (NK cell) and adaptive (T-cell) immune programs.
3. Employ proteomic and cytokine array analysis to obtain a broader understanding of the NR mediated effects on mitochondrial biology in modulating immune regulation in circulating cells and in the serum of study subjects.

5. Study Design and Procedures

The study design is a prospective, double-blind randomized crossover study with subjects serving as their own controls.

Normal volunteers will be enrolled on this 3-5 week study that includes 3 visits to the NIH Clinical Center. Subjects may be contacted by telephone, text or email to verify study compound compliance, for AE/safety assessment and to remind subjects to initiate the 24-hour fast prior to Visit #2 and Visit #3. Email communications will be made through the NIH medical secure email system unless the patient declines its' use and requests standard unsecure email. A schematic showing the outline of the overall study is shown below.



The variable length of the washout and study arms phase is to allow flexibility for the scheduling of subject visits. The prior pharmacokinetic studies support that 7 days is more than sufficient to restore NAD^+ levels to baseline.

The following actions will be undertaken at the defined visits:

Arm 1

Screening/Visit 1:

- Subjects will be screened for inclusion or exclusion.
- Baseline screening laboratories performed.
- Pregnancy testing in women of childbearing potential.

- Eligible subjects will be randomized to placebo (i.e. sugar tablet) or 1 gram of NR daily. The study compound start date will be determined at this visit. Start date will be within one week of screening visit.
- Instruct subject on study compound administration, reinforce maintenance of stable diet and activity level during the study participation period, and review the time of the breakfast on day 6 (+3 days, if needed), prior to initiating the 24-hour fast.

Day 1 of Arm 1 study compound administration.

A member of the research team may contact study subjects:

- to remind them which day to initiate therapy
- to remind subjects of the 24-hour ± 2hr fast

Visit 2 : Day 7 (+ up to 3 days, if needed) of Arm 1 study compound administration

- Subjects will arrive at the Clinical Center after a 24-hour fast ± 2 hours with the exception of drinking water starting the morning of day 6 (+ up to 3 days, if needed).
- A pill count will be performed to evaluate compliance.
- Fasting blood draw for hepatic panel, acute care panel, insulin, growth hormone, complete blood count, serum immune cell studies and to measure NAD levels.
- Urine collection for ketones
- Administer day 7 (+ up to 3 days, if needed) Arm 1 placebo/NR capsules.
- A 500 caloric meal will be eaten.
- Refed blood-draw for insulin, growth hormone, glucose, serum immune cell studies and to measure NAD levels three hours ± 30 minutes after fixed caloric meal (metabolic kitchen).
- Subject educated about washout period.
- New bottles of study compound for Arm 2 dispensed.

Washout period of 7-14 days

Arm 2

Day 1 of Arm 2 study compound administration

A member of the research team may contact study subjects:

- to remind them which day to initiate therapy
- to remind subjects of the 24-hour ± 2hr fast

Visit 3: Day 7 (+ up to 3 days, if needed) of Arm 2 study compound administration

- Subjects will arrive at the Clinical Center after a 24-hour fast ± 2 hours with the exception of drinking water starting the morning of day 6 of the second study arm.
- A pill count will be performed to evaluate compliance.
- Fasting blood draw for hepatic function panel, acute care panel, complete blood

count, insulin, growth hormone, serum immune cell studies and to measure NAD levels.

- Urine collection for ketones
- Administer day 7 of Arm 2 placebo/NR capsules.
- A 500 caloric meal will be eaten.
- Refed blood draw for insulin, growth hormone, glucose, serum immune cell studies and to measure NAD levels three hours ±30 minutes after fixed caloric meal (metabolic kitchen).

Off Treatment (Supplement):

Subjects will be considered off treatment (supplement) after completion of Arm 2 study pills.

Follow up:

We will follow up the subjects for 30 days after completion of Arm 2 study pills for any AEs. Subject will be instructed to the contact research team if any symptoms develop during this 30 day period.

Off Study:

Subject will be taken off study 30 days after completion of Arm 2 study pills. If a subject doesn't report any AEs, we will take him/her off study on day 30 after completion of Arm 2 study pills. If a subject reports any AEs during the 30-day follow up period then we will adjust off study date upon resolution/returning to the baseline of the AEs.

Randomization and dispensing of study pills:

The pharmacist Co-Investigator on the study will devise a randomization code in consultation with Dr. Myron Waclawiw (Biostatistician). This code will be kept confidential and will be employed by the pharmacy to dispense the study pills. For each arm, subjects would receive sufficient pills for 7 days of administration. If additional pills are needed to extend the treatment arm by up to three days, these will be dispensed separately by the pharmacy. Subjects will take their study medication at home for days 1 through 6 (+ up to 3 days, if needed). Day 7 (+ up to 3 days, if needed) dosing will be in the clinic. Subjects will be instructed to bring their study medication bottle back to the clinical center for day 7 (+ up to 3 days, if needed) dosing and to not take the day 7 (+ up to 3 days, if needed) dose until instructed to by the study team. If the subject forgets to bring the study bottle with the day 7 (+ up to 3 days, if needed) dose, the day 7 (+ up to 3 days, if needed) dose will be ordered through CRIS from the pharmacy. A process has been developed by the NHLBI pharmacist to ensure the subject receives the correct study compound or placebo. The day 7 (+ up to 3 days, if needed) pill for each arm will be given to the study subject for consumption following the 24-hour fasting research blood draw. The study pill receptacle will have the subject's name, code number, prescription details (4 tablets by mouth each morning with or without food) and will include the arm (1 or 2). If the study subject does not bring in the pill bottle, the subject will be asked to confirm the number of study doses taken over the prior 6 (+ up

to 3 days, if needed) days and this will be documented in the medical record. The pill bottles will then either be collected at the next visit or we will have the subject return by FedEx.
The code will be shared with the statistician following the completion of studies on 20 subjects to evaluate observed standard deviations to assess original study assumptions and continued study feasibility.

Description of Study Population and Recruitment

Participants will be enrolled at the NIH Clinical Center. Subjects will be recruited through a LISTSERV email announcement and study procedures will be performed in the outpatient unit/day hospital at the NIH Clinical Center. A list of healthy volunteers may also be requested from the NIH Office of Patient Recruitment.

A total of 42 participants will be recruited for this study.

6. Eligibility Assessment

The PI and/or AI will assess eligibility. Screening will be performed under this protocol only. Screening studies for subject enrollment:

i. History and physical examination.
ii. Screening and Research laboratory tests may include:
    - Acute Care Panel (Na, K, Cl, CO2, Creatinine, Glucose, and Urea Nitrogen)
    - Mineral Panel (Phosphorus, Magnesium, Albumin, and Calcium)
    - Hepatic Panel (Alk Phosphatase, ALT, AST, Total Bilirubin, and Direct Bilirubin)
    - CBC + Differential
    - Blood draw for immune cell studies and NAD levels
    - Urine for ketones
    - Growth hormone ○

    Insulin level
    - Glucose
    - βHCG (females of childbearing potential)

Inclusion Criteria
As this is a pilot study, the age-range and BMI range of subjects will be restricted to potentially reduce metabolic variables associated with a wide age- and BMI-range.

- Males and females between the ages of 18 and 39
- BMI between 18.5 and 29.9
- Agrees to comply with study procedures and maintain current level of physical activity and dietary intake throughout the study.
- Female subjects of child-bearing ability willing to commit to reliable contraception while participating in the study.

Exclusion Criteria

- Subjects with an acute or chronic illness as per history, on laboratory analysis or requiring medications to manage disease.
- Subjects taking vitamins or supplements or any medications, except oral contraceptives, within 4 weeks of participation into this study.
- BMI <18.5 or >29.9.
- Female subjects who are pregnant or lactating.
- Subjects who have donated blood or participated in another clinical trial involving blood draws in the last 8 weeks.
- Subjects who use nicotine products including chewing tobacco, vaporizer, gum, cigarette or patch form within three months.
- Any other medical condition that, in the opinion of the Principal Investigator, might put the subject at risk of harm during the study or might adversely affect the interpretation of the study data.

7. Procedures

All tests will follow NIH Clinical Center policies. Any of the listed procedures or laboratory tests performed within three months prior to enrollment in this protocol may/may not be repeated. However, if these procedures are not repeated, the prior studies (within the last 3 months) will be reviewed in CRIS as part of the protocol. The purpose for not repeating study procedures is to prevent unnecessary inconveniences, risks or discomforts for study participants and the NIH Clinical Center.

i) ***Blood Draws:*** In addition to the screening blood draw (40 mL), subjects will have 4 additional research blood draws up to 100 mL each, as described in Section 4.

ii) ***Urine Analysis:*** Subjects will be asked to provide up to three urine samples, one for pregnancy testing as part of screening evaluation and two in the fasting state at Visits 2 and 3 as described in Section 4.

iii) ***24-Hour Fasting:*** Subjects will be asked to fast for up to 26 hours (water intake will not be restricted) during each of the two treatment arms.

iv) ***Fixed Caloric Meal:*** The meal will be a 500 calorie breakfast. Subjects will have a choice of two meals.

Meal Choices	Description of Meal
Option 1	• Vegetable omelet • Toast with butter and jelly • Orange juice
Option 2 -	• Oatmeal with Walnuts, Brown Sugar and Dried Cranberries • Milk

8. Dietary Supplement

Niagen™ is a commercially-available form of nicotinamide riboside (NR). The nucleoside NR is a single chemical moiety containing nicotinamide and ribose.[7] NR is a form of vitamin B_3 present in trace amounts in foods like milk, yeast extract and beer. It is also postulated that NR is generated in the gastrointestinal tract as part of dietary NAD+ digestion. Thus, humans are constantly exposed to NR from the diet, albeit at low levels. Since 2013, Niagen™ has been sold as a dietary supplement in the United States. Labeling guidelines recommend consumers to limit their intake to 2 capsules/day, which amounts to 250 mg/day. This recommended level is the equivalent of 3.8 mg/kg bw/day, which is 1000-fold less than the highest dose determined to be safe and well tolerated in rats, and a quarter of the dose proposed for this pilot clinical trial. There is no limit on the duration of ingestion. Limited animal model and human data exist on the pharmacokinetics and safety of orally administered NR. However, ChromaDex has conducted a recent randomized, double-blind, cross-over, pharmacokinetic study (14NBHC, unpublished results) which demonstrated that NR was readily absorbed and detectable in human plasma, white blood cells, and urine. In addition, NR was well tolerated and presented no toxicity following a single dose up to 1000 mg in human subjects. Moreover, in an acute toxicology study, rats that were given a single oral dose (5000 mg/kg) of NR did not show clinical signs of toxicity or mortality (unpublished results).

Niacin is a form of vitamin B_3[8] and has been used for a long time treat hypercholesterolemia and pellagra. Niacin administration can lead to undesirable effects, such as spontaneous flushing. Studies in animal models suggest that flushing results from nicotinic acid-mediated activation of the G-coupled receptor, GPR109A.[9] NR has low affinity for GPR109A, and experiments in cell lines expressing GPR109A demonstrate that nicotinic acid, but not NR, activates the receptor. [2] Thus, based on these findings, oral ingestion of NR may not result the spontaneous flushing that has been associated with high doses of niacin administration.

Multiple lines of non-clinical data suggest that NR intake does not present any potential risks and should be well tolerated in human subjects. The dose proposed for this study is within the doses tested in non-clinical studies of mice for up to 4 months, and are 300-fold below the daily dose that was given in rats in a 14-day dose range finder study.

Nicotinamide, an expected metabolite of NR, is a molecule that is considered of low toxicity in food by several regulatory agencies including the United States Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA).

Because nicotinamide is a putative metabolite of NR in humans, understanding the safety profile of nicotinamide is relevant when assessing the safety of NR in human subjects.

Based on the structural similarities between NR and nicotinamide, and that nicotinamide is a downstream metabolite of NR digestion, it is assumed that any unexpected adverse effects of NR may be similar to those associated with nicotinamide intake. However, no significant adverse

effects have been reported in clinical trials which have used doses up to the equivalent of 3000 mg/day for up to 3 years to evaluate the possible benefits of nicotinamide administration to patients with or at risk of developing Type 1 diabetes [10, 11]. In addition, doses of 25 and 42 mg/kg bw/day had no effect on a variety of biochemical parameters, such as those that assessed liver and kidney function.

In summary, careful analysis of all the non-clinical information available on NR has not revealed any potential serious toxicity that would preclude its use in healthy subjects.

Common name: Nicotinamide Riboside Chloride

Product name: Niagen

Chemical name: 3-(Aminocarbonyl)-1-β-D-ribofuranosyl-pyridinium chloride (1:1)

Dose: 1000mg daily for 7-10 days

Route of administration: oral

Dosing instructions: (4) 250mg study capsules daily after breakfast.

Supply: Drug will be supplied by ChromaDex

Manufactured by: W.R. Grace & Co. 1290 Industrial Way, Albany, OR 97322, USA

Toxicology: None known.

Drug Interactions: None known.

Off-label use: Considering the clinical investigation is designed to study the relationship between a dietary supplement's effect on normal structure or function in humans or to characterize the mechanism by which a dietary supplement acts to maintain such structure or function this study would not need to be conducted under an IND. Under the Dietary Supplement Health and Education Act of 1994, a dietary supplement is not considered a drug and is not subject to the premarket approval requirements for drugs if the intended use for which it is marketed is only to affect the structure or any function of the body (i.e., not intended to be used for a therapeutic purpose). Similarly, whether an IND is needed for a clinical investigation evaluating a dietary supplement is determined by the intent of the clinical investigation. If the clinical investigation is intended only to evaluate the dietary supplement's effect on the structure or function of the body, an IND is ***not*** required.

9. Data and Biospecimen Management Plan

Data Management and Access:

Samples will be de-identified prior to storage on the 5th floor of building 10 in laboratory for the principal investigator following current NIH sample storage guidelines. Samples and data will be stored, using codes assigned by the investigators or their designee(s). Research samples will be stored using BSI in accordance with NHLBI DIR Biospecimen policy. Data will be kept on the NHLBI P:drive, accessible through password-protected computers. Only the members of the research team will have access to the samples and data.

Primary research data will be coded by replacing individually identifying information (such as name) with a code that will enable the investigator to readily ascertain the identity of the subject through the use of a code-key, but will not reveal the identity of subjects to parties not authorized to have access to individual subject identifiers.

Biospecimen Management:

Blood and urine samples will be coded and stored in conformity with DIR Policy (e.g., BSI). Coded biospecimens may be sent to collaborators outside of the NIH with IRB approval in accordance with applicable NIH and DIR Policy for sharing research resources, including an executed material transfer agreement.

End of study procedures:

Data retained by the NHBLI will be stored in a password-protected database in conformity with NHLBI DIR policy until they are no longer of scientific value.

Destruction of research data collected on this protocol will be consistent with NIH policy and upon permission of the Clinical Director.

Breach of Confidentiality:

PIs will report any breach of subject confidentiality or trial data to the clinical director and IRB per NIH policy, including NIH HRPP SOP 16 - Reporting Requirements for Unanticipated Problems, Adverse Events and Protocol Deviations.

Data Sharing and Future Use of Data Data

Sharing Plans:

Research data may be shared with qualified non-collaborator recipients following publication of the primary research results after removal of PII and IRB approval (coded data) or Office of Human Subjects Research Protections (OHSRP) approval (unlinked data). In both situations, a data use agreement between the sender and the recipient will be executed. Future research use of data not defined in the research protocol may occur only after IRB review and approval or a determination from the NIH OHSRP. Refusal of a research subject participant to permit future use of data-will be honored.

Future Use of Biospecimens:

Following analyses of biospecimens for primary research purposes as described in the protocol, remaining samples suitable for future research will be stored in manner that conforms with DIR policy (such as BSI) or in a publicly accessible research biospecimen repository following IRB approval. Biospecimens may be destroyed only when permitted by the clinical director and approved by the IRB. Any future research use of biospecimens not defined in the research protocol will occur only after IRB review and approval, if the research holds the key that identifies research subjects, or determination from OHSRP (non-collaborative research). Biospecimens will not be sent outside of the NIH for future research use without IRB approval and an executed agreement. Refusal of a research subject participant to allow for future use of biospecimens will be honored.

Loss or destruction of samples: Should we become aware that a major breech in our plan for tracking and storage of samples has occurred, the IRB will be notified.

Collaboration:

Between NHLBI and Dr. Julian L. Griffin, Department of Biochemistry, Cambridge University, Cambridge, UK. This MTA is for the NHLBI to send de-identified blood and serum samples from healthy volunteers from this fasting and refeeding study for lipidomic and metabolomic testing. An MTA will be executed after Amendment D approval by the IRB.

10. Statistical Considerations

We propose that NR functions as a fasting mimetic and we will assess whether after refeeding the NLRP3 inflammasome activation (IL-1β release) is blunted by NR as the primary endpoint. In our prior study, the mean±SD value of fasting IL-1β release was 319±513 pg/ml, while the mean refed value was 736±1067 pg/ml (with 417±613 pg/ml for the paired refed-fasting IL-1β release differences). In this placebo-controlled crossover study, we would like to detect a relative reduction of at least 20% in refeeding IL-1β release (from 736 to 589 pg/ml) with NR treatment compared to the paired responses on placebo. The crossover design has the benefit of reducing the influence of confounding covariates (because each subject serves as their own control) and is statistically efficient, requiring fewer subjects than non-crossover designs. Since estimates of within-subject variability for both the NR and placebo arms, between-subject variability for the NR arm, and between-subject covariance between NR and placebo are not available for this novel exploratory study, the usual crossover study sample size formulas cannot be used. Instead, we calculate sample size using the paired Student t-test. With a standard deviation of 276 pg/ml (45% of that observed for the fasting-refed differences), 80% power and an alpha=0.05, we will need 30 subjects to detect a 20% relative decrease (absolute decrease of 147 pg/dl from 736 to 589 pg/dl) for the paired treatment differences in IL-1β release with NR treatment. We therefore request approval to enroll up to 35 normal volunteers to account for possible treatment non- compliance and /or study dropouts.

There will be no interim efficacy look at the data in this proof-of-concept study. However, when 20 subjects have completed the study, the protocol statistician will perform a sample size re-

calculation using only the observed standard deviation of the paired between treatment IL-1β changes to assess the original study assumptions and continued study feasibility. The study PI will remain blinded to the primary outcomes. The study will be judged feasible jointly by the study PI and Statistician if the new sample size calculation shows that 50 or fewer subjects can answer the primary hypothesis concerning IL-1β. If the study is feasible and continues, the sample size will not be reduced below the originally calculated requirement of 30 but, if needed, a request for a new larger enrollment ceiling (not to exceed 58 subjects, accounting for study drop-outs and non-adherers) will be presented to the IRB for consideration.

Of note, the sample size recalculation was performed on October 19th, 2017 after the enrollment of 20 subjects. The study sample size was recalculated using an updated observed standard deviation of the between treatment fed-fasting differences in IL-1beta levels (S = 177.002 pg/ml) based on 17/20 evaluable subjects enrolled into the protocol. The new calculation was performed for fed-fasting differences rather than fed differences between treatments (a more conservative approach) to allow adjustment for any differences in baseline fasting IL-1beta levels between study periods. With an observed standard deviation of 177 pg/ml, 80% power, and a two-sided alpha=0.05, we will need n=36 subjects to detect a 20% relative decrease (absolute decrease of 83 pg/ml from the protocol-specified mean level of 417 pg/ml to 333 pg/ml) for the paired fed-fasting differences between treatments in IL-1beta release with NR treatment. As the study continues to be feasible (requires the enrollment of fewer than 50 subjects), we request approval to continue enrolling up to 42 normal volunteers to account for possible treatment non-compliance and/or study dropouts.

Primary study outcome:

The primary study outcome will be the refeeding IL-1β secretion in response to inflammasome stimulation in the NR treatment arm versus the same subject's response on the placebo treatment arm using the nonparametric Wilcoxon Signed Rank test.

Additional Analyses:

- NLRP3 inflammasome activation in PBMC's (IL-1β in the fasting state)
- Isolate PBMC's to explore mitochondrial functioning and biochemistry (oxygen consumption rate in leukocytes)
- Flow cytometry to explore innate and adaptive cell profiles (assessment of cell surface markers to assay cellular polarity and activation)
- Negative selection to extract T-cells to investigate primary culture T-cell polarity (ELISA assays to measure cytokine production following T-cell activation)
- Serum samples for SomaLogic Proteome analysis and Luminex analysis of cytokine levels (discovery proteomics data collection)
- ELISPOT analysis of $T_H1/T_H2/T_H17$ cytokine polarity following TCR crosslinking (Alternate assay to measure CD4 T-cell polarity)

In this pilot study, the parametric paired Student t-test and the non-parametric Wilcoxon Signed Rank test will be used to analyze treatment differences in secondary outcome measurements.

11. Stopping Rules for Subjects

- Pregnancy and breastfeeding
- Subjects taking less than 75% of the supplement
- Any other significant medical symptoms that may or not be related to the treatment and as determined by contact with the physician
- Subjects who are found to be pregnant or wish to breastfeed during the study will automatically be withdrawn. A pregnancy screening will be done for all female volunteers at the first visit and subjects will be excluded from the study if pregnant

12. Data and Safety Monitoring

Safety Monitoring

Accrual and safety data will be monitored by the Principal Investigator, Michael N. Sack, M.D. Ph.D., who will provide oversight to the conduct of this study.

Accrual and safety data will be monitored and reviewed annually by the Institutional Review Board (IRB). Prior to implementation of this study, the protocol and the proposed subject informed consent document will be reviewed and approved by the properly constituted IRB operating according to the 45 CFR 46. This committee must approve all amendments to the protocol or informed consent document, and conduct continuing annual review so long as the protocol is open to accrual or follow up of subjects.

Adverse Event Reporting

Adverse Event (AE): Any untoward medical occurrence in a human subject, including any abnormal sign (e.g., 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.

Abnormal laboratory values: A moderate and severe abnormal laboratory value will be considered an AE if the laboratory abnormality is characterized by any of the following:

- Results in discontinuation from the study
- Is associated with clinical signs or symptoms
- Requires treatment or any other therapeutic intervention
- Is associated with death or another serious adverse event, including hospitalization
- Is judged by the Investigator to be of significant clinical impact
- If any abnormal laboratory result is considered clinically significant, the investigator will provide details about the action taken with respect to the subject's outcome.

***Serious Adverse Event (SAE)*:** A serious adverse event that:

- results in death;
- is life-threatening (places the subject at immediate risk of death from the event as it

occurred);

- results in in-patient hospitalization or prolongation of existing hospitalization;
- results in a persistent or significant incapacity;
- results in a congenital anomaly/birth defect; or
- based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition.

Unanticipated Problem: An UP is any incident, experience, or outcome that meets all of the following criteria:

1. **unexpected** in terms of nature, severity, or frequency in relation to

a. the research risks that are described in the IRB-approved research protocol and informed consent document; Investigator's Brochure or other study documents; and

b. the characteristics of the subject population being studied; and

2. **related or possibly related** to participation in the research; and

3. places subjects or others at a **greater risk of harm** (including physical, psychological, economic, or social harm) than was previously known or recognized.

Reporting of Pregnancy:

In the event a subject becomes pregnant while on study, this event will be reported to the IRB and Clinical Director as an unanticipated problem. Monitoring of the pregnancy will continue until conclusion of the pregnancy.

Unanticipated Problem that is not an Adverse Event: An unanticipated problem that does not fit the definition of an adverse event, but which may, in the opinion of the investigator, involves risk to the subject, affect others in the research study, or significantly impact the integrity of research data. For example, report occurrences of breaches of confidentiality, accidental destruction of study records, or unaccounted-for study drug.

Protocol Deviation (PD): Any change, divergence, or departure from the IRB approved research protocol.

Non Compliance: The failure to comply with applicable NIH HRPP policies, IRB requirements, or regulatory requirements for the protection of human research. Noncompliance may be further characterized as:

Serious non-compliance: Non-compliance that:

a. Increases risks, or causes harm, to participants.
b. Decreases potential benefits to participants.
c. Compromises the integrity of the NIH HRPP.
d. Invalidates the study data.

Continuing non-compliance: Non-compliance that is recurring. An example may be a pattern of non-compliance that suggests a likelihood that, absent an intervention, non-

compliance will continue. Continuing noncompliance could also include a failure to respond to IRB requests to resolve previous allegations of non-compliance.

Minor (non-serious) non-compliance: Non-compliance that, is neither serious nor continuing.

Adverse Event Management

The principal investigator and medical advisory investigator, or designee, will be responsible for assessing adverse events. Information on adverse events will be solicited from subjects through questions from study personnel and/or information volunteered by the subject. Adverse events will be captured from the start of the first pill taken (day 0) until 30 days following the last pill taken. Following this period, only serious adverse events that are unexpected and related to study pills will be reported. All AEs, regardless of severity, will be recorded, graded, assigned an attribution, verified, and followed until satisfactory resolution.

In the event of any treatment-related SAEs, enrollment will be suspended until discussed with the IRB and Clinical Director.

Grading and Attribution of Adverse Events

Mild: Awareness of sign or symptom, but easily tolerated. Not expected to have a clinically significant effect on the patients overall health and well-being. Not likely to require medical attention.

Moderate: Discomfort enough to cause interference with usual activity or affects clinical status. May require medical intervention.

Severe: Incapacitating or significantly affecting clinical status. Likely requires medical intervention and/or close follow-up.

Attribution of Adverse Events

Relationship	**Attribution**	**Description**
Unrelated to intervention	Unrelated	The AE *is clearly **NOT** related* to the intervention
	Unlikely	The AE *is **doubtfully related*** to the intervention
Related to intervention	Possibly	The AE ***may be related*** to the intervention
	Probably	The AE ***is likely related*** to the intervention
	Definitely	The AE ***is clearly related*** to the intervention

NHLBI-IRB and CD reporting

Serious Events

Reports to the IRB and CD: The PI must report Serious UPs, and Serious PDs to the IRB and CD as soon as possible but not more than 7 days after the PI first learns of the event using the NIH Problem Report Form.

Non-serious Events

Reports to the IRB and CD: The PI must report all UPs that are not Serious to the IRB and CD, and PDs that are not Serious to the IRB, not more than 14 days after the PI first learns of the event using the NIH Problem Report Form.

Deaths

Deaths possibly, probably, or definitely related to study procedures will be reported to the Clinical Director within 7 days after the PI first learns of the event.

Reports at the time of continuing IRB review: At continuing review, the PI will provide to the IRB a summary of:

- All UPs
- All PDs
- All AEs (except for those granted a waiver of reporting)

Waiver of Reporting: The following adverse events will be listed in the consent and not reported to the IRB:

- Vasovagal symptoms during blood draws (expected frequency 50%).
- Transient bruising at the site of blood draws (expected frequency 50%).
- Hunger, headache, fatigue, or irritability during fasting (expected frequency 50%).

13. Human Subjects Protections

Rationale for Subject Selection

Subjects of both genders will be considered for inclusion in this study. There will be no racial, ethnic, or gender discrimination. Cognitively impaired and institutionalized persons will not participate in this study. Recruitment, enrollment and compensation of NIH employee subjects will be consistent with NIH Manual Chapter 2300-630-3: Leave Policy for NIH Employees.

Rationale for the Exclusion of Children

Subjects under 18 years of age will not be considered for inclusion in this protocol because there is no direct benefit from participating in this study and the 24-hour fast and volumes of blood levels drawn exceed minimal risk for children.

Rationale for the Exclusion of Pregnant Women

Subjects must not be pregnant or actively seeking pregnancy in order to participate in this study. NR has not been determined to be safe in pregnancy or breastfeeding. A recognized form of contraception must be used by subjects while enrolled. Contraception use will be determined during telephone screening and confirmed at the screening visit.

Rationale for the Exclusion of Cognitively Impaired Subjects

Subjects with cognitive impairment will not be considered for inclusion because there is no direct benefit from participating in this study.

Risk/Benefit Assessment

The research involves greater than minimal risk to subjects, with no prospect of direct benefit, but is likely to yield generalizable knowledge (45 CFR 46.102).

Inclusion of NIH Staff

- NIH staff (employees, NIH contractors, special volunteers, guest researchers, and trainees) may voluntarily participate in this protocol.

• Recruitment, enrollment and compensation of NIH staff will be consistent with the Guidelines for the Inclusion of Staff in NIH Intramural Research Studies (December 2015) (Appendix A of SOP 14F) and NIH Policy Manual Chapter 2300-630-3,:"Leave Policy for NIH Employees Participating in NIH Medical Research Studies" (Appendix B of HRPP SOP 14F,).

If the individual requesting to participate in the protocol is a co-worker, the consent from the NIH staff member (co-worker) will not be obtained by the staff member's direct supervisor but by another research staff member approved for obtaining informed consent, and who is also not a co-worker.

• Neither participation nor refusal to participate as a subject in this protocol will have an effect, either beneficial or adverse, on the participant's employment or position at NIH. However, all subjects will be made aware that there are limits to these protections.

• The PI, through the consenting staff member will make the "NIH Information Sheet on Staff Research Participation" available to staff members who are considering enrolling in research. (SOP 14F, Appendix C; also in Appendix A of the protocol)

Risks and Discomforts

- ***Phlebotomy:*** Standard precautions for obtaining human blood samples will be taken. Transient discomfort and minor bruising may occur at the phlebotomy site. Vasovagal symptoms can occur during blood drawing. Blood samples will be obtained by venipuncture. Blood samples will be obtained by a nurse, physician, or other skilled individual. A total of 440 mL of blood with be drawn for this study. The quantities of blood to be drawn for research purposes will be consistent with the CC policy as provided in Medical Administrative Series (MAS) 95-9 (revised 05/29/2012):
  a. Subjects 18 years of age or older (index cases and relatives): It is estimated that 100 mL will be drawn in one blood draw (but never exceeding 10.5 mL/kg or 550 ml (whichever is smaller) over any 8-week period.
- ***24-Hour Fast:*** Subjects may experience hunger, headache, fatigue, or irritability during fasting. Subjects will be instructed not to take Acetaminophen (Tylenol®).

Acetaminophen is usually well tolerated in prescribed dose, but when taken during fasting may cause of drug-induced liver disease and acute liver failure. The first symptoms of liver damage are seen in the initial 12 to 24 hours or so after ingestion and include nausea and vomiting.

- ***Urine collection:*** There is no risk associated with this procedure.

Nicotinamide Riboside: NR is a dietary supplement that is currently available for commercial use with no safety concerns noted to date. Consecutive doses of 1000mg daily of NR has not been reported in humans although there is currently an ongoing study at the University of Colorado using 1000 mg daily for 6 weeks. Based on the structural similarities between NR and nicotinamide, and that nicotinamide is a downstream metabolite of NR digestion, it is assumed that any unexpected adverse effects of NR may be similar to those associated with nicotinamide intake. The safety profile of nicotinamide has been well established in multiple species and provides reasonable certainty that the administration of doses of NR up to 1000 mg will not result in an adverse health effect. No significant adverse effects have been reported in clinical trials which have used doses up to the equivalent of 3000 mg/day for up to 3 years to evaluate the possible benefits of nicotinamide administration to patients with or at risk of developing Type 1 diabetes.[10, 11] In addition, doses of 25 and 42 mg/kg bw/day had no effect on a variety of biochemical parameters, such as those that assessed liver and kidney function.

Consent Processes and Documentation for Research Subjects

Each participant will receive an oral and written explanation of the goals, procedures, and risks of this study. The Principal Investigator and those Associate Investigators who are listed on the cover page of the protocol with an asterisk next to their name may obtain informed consent from research participants. Consent will be obtained at the NIH Clinical Center. The original, signed informed consent document will be placed in the medical record, and the subject will receive a signed copy of the informed consent document.

If there is an unexpected enrollment of a non-English speaking research participant for which there is no translated extant IRB approved consent document, the principal investigator and/or those authorized to obtain informed consent will use the Short Form Oral Consent Process as described in MAS Policy M77-2, 45 CFR 46.117 (b) (2). The summary that will be used is the English version of the extant IRB approved consent document.

We request prospective IRB approval of the use of the short form for up to a maximum of 2 participants in a given language and will notify the IRB at the time of continuing review of the frequency of the use of the Short Form. Should we reach the threshold of 2, we will notify the IRB of the need for an additional use of the Short Form and that we will have that consent document translated into the given inherent language.

Consent Processes and Documentation for NIH Staff

If the individual requesting to participate in the protocol is a co-worker, the consent from the employee (co-worker) will not be obtained by the research coordinator or the staff member's direct supervisor but by another research staff member who is approved for obtaining informed consent, and who is also not a coworker. NIH staff will be given the NIH Information Sheet on *Staff Research Participation* (See appendix A) to help them understand the possible consequences of participation.

14. Conflict of Interest

None of the members of the research team reported a potential conflict of interest. The National Institutes of Health reviews NIH staff researchers at least yearly for conflicts of interest. The following link contains details on this process http://ethics.od.nih.gov/forms/Protocol-Review-Guide.pdf. This protocol has a CRADA with ChromaDex, Inc.

15. Reimbursement for Travel

As the study populations will be local, reimbursement for travel, food, and lodging will not be provided.

16. Financial Compensation

Subjects will be compensated for procedures that are performed that are of no direct benefit to the subject, although would be of generalizable benefit to enhance our understanding of Participants will be compensated as described in the table below:

Procedures	Inconvenience Units	Compensation per procedure	Frequency	Total Compensation
Medical History and physical examination	2.5	$25.00	1	$25.00
Outpatient Visit (first hour)	2	$20.00	3	$60.00
Outpatient Visit (additional hours up to 4 hours)	1	$10.00	8	$80.00
Screening Blood Draw (if needed)	1	$10.00	1	$10.00
Research Blood Draw	2.5	$25.00	4	100.00
24-Hour Fast	15	$150.00	2	$300.00
Urine Sample	1	$10.00	3	$30.00
Drug administration, General	2	$20.00	14	$280
			Maximum Compensation:	$885.00

17. References

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APPENDIX A: NIH INFORMATION SHEET ON STAFF RESEARCH PARTICIPATION (APRIL 2016)

As an NIH employee, contractor, Special Volunteer, Guest Researcher, or trainee, you may participate in intramural research studies unless it is prohibited by your Institute or Center (IC), or if you are excluded by the criteria of the protocol in which you want to enroll. The inclusion of NIH staff in a particular protocol must also be approved by the IRB. You may be motivated by altruism, a commitment to research in your own or related fields, or want access to clinical trials of potential direct therapeutic benefit. When deciding, you should make an informed decision about participation. This information sheet offers some points to consider for NIH staff who are considering research participation at NIH.

First, similar to any individual who is considering research participation, you should seek adequate information about the study purpose, what is required of you in terms of procedures, interventions and time, and the potential risks and benefits of participation. For more information, see the NIH Clinical Center's public website "Are Clinical Studies for You?" at http://www.cc.nih.gov/participate/studies.shtml.

When you are thinking about participation in a research study that is being conducted by your supervisor, or others with whom you work closely in your laboratory, branch, or unit, you should consider some additional factors:

A. **Possible bias:** Are you confident that you can be unbiased about reporting answers, side effects, or other information that could influence the study outcome or risk to you?

B. **Confidentiality:** Has the principal investigator (PI) spoken about what information will be collected from you as part of the study? Has the PI discussed what information will be available to those within, and outside, the study team? If applicable, are you comfortable sharing your medical history (including, for example, mental health history or STDs) and your social history (e.g. substance use) with study investigators who may be your coworkers, or with the possibility of them discovering something about your health during the study (e.g. pregnancy status or a new diagnosis)? Although every effort will be made to protect your information and keep it private and confidential, your information may, depending on the nature of the protocol, become available in medical records or to authorized users outside of the study team. Discuss any concerns with the PI..

C. **Pressure**: Do you perceive any pressure or expectations from your supervisor or colleagues regarding participation? Could that pressure influence your decision or make it difficult for you to choose whether or not to participate? Remember that it is your choice whether or not to participate and that your decision to participate or not should not have an effect, either beneficial or adverse, on your position at NIH.

D. **Time and Compensation:** Can you take time off from work to complete the study requirements or participate solely during non-duty hours? Can you receive compensation for your participation in this study? Will your supervisor give you permission to participate during

work hours? See the NIH Policy Manual 2300- 630-3 Leave Policy for NIH Employees Participating in NIH Medical Research Studies.

E. **Consent Process:** Is the person obtaining your consent for the study your supervisor, a subordinate, or co-worker? If so, is there an independent person monitoring the consent process? If the study PI is a supervisor and intends to obtain consent from you, an independent person (e.g., through Bioethics or the NIMH Human Subjects Protections Unit [HSPU], or others as approved by the IRB) must monitor the consent process. If the person obtaining consent from you is a co-worker then an independent person (e.g., through Bioethics or the NIMH HSPU, or others as approved by the IRB) may be required to monitor the consent process, as determined by the IRB for the specific study.

If you are thinking of enrolling as a subject at the NIH Clinical Center and you have any questions or concerns, please contact the Office of Human Subjects Research Protections (OHSRP) at 301-402-3444 and/ or the Patient Representative if you are thinking of enrolling as a subject at the NIH Clinical Center on 301-496-2626. If you are at a NIH site outside the Clinical Center then please contact local site leadership.